

**EXPERIMENTAL INVESTIGATION OF THE CONTROL AND FUNCTION
OF THE *MUSCLE SEGMENT HOMEBOX* GENES DURING
VERTEBRATE EMBRYOLOGICAL DEVELOPMENT.**

JON MARTIN COLLINSON

Ph.D.

UNIVERSITY OF EDINBURGH

MRC HUMAN GENETICS UNIT

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DECLARATION

I declare that this thesis was composed by myself and that the work described within it is my own, except where stated.

Martin Collinson
September 1995

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To all the mice and chickens:

I'm sorry; I won't hurt you anymore. I dream that we will meet again and share crumbs around a table in the farmhouse kitchen, which is Paradise.

" I am the family face;
Flesh perishes, I live on,
Projecting trait and trace
Through time to times anon,
And leaping from place to place
Over oblivion. "

Thomas Hardy - 'Heredity'

ABBREVIATIONS

A	Adenine
AER	Apical ectodermal ridge
ANZ	Anterior necrotic zone
β -Gal	β -Galactosidase
BMP	Bone Morphogenetic Protein
bp	Base pair
C	Cytosine
cDNA	Coding DNA
Ci	Curies
CNS	Central nervous system
CRABP	Cellular retinoic acid binding protein
DIG	Digoxigenin
DMEM	Dulbecco's Modification of Eagle's Medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
DTT	Dithiothrietol
E	Embryonic day
FCS	Foetal calf serum
G	Guanine
g	grams
G418	Geneticin 418
INZ	Interdigital necrotic zone
kb	kilobases
kD	kiloDaltons
mRNA	messenger RNA
<i>neo</i> ^r	<i>neomycin resistance</i>
NIH	National Institute of Health
PBS	Phosphate-buffered saline
PNZ	Posterior necrotic zone
PZ	Progress Zone
RA	Retinoic acid
RAR	Retinoic acid receptor

RXR	Retinoid-X receptor
RNA	Ribonucleic acid
rpm	Revolutions per minute
s	seconds
SDS	Sodium dodecyl sulphate
ssDNA	Single-stranded DNA
T	Thymine
TGF	Transforming Growth Factor
UTR	Untranslated region
UV	Ultraviolet
W	Wanek <i>et al.</i> (1989) limb stage
wt	Wild-type
ZPA	Zone of polarising activity

ABSTRACT

The control and function of the *msh*-class (*muscle segment homeobox*) genes, *Msx1* and *Msx2*, was investigated during vertebrate embryonic development. These genes are widely expressed in the embryo, often in structures which are elaborations on the presumed ancestral chordate body-plan. It is thought that duplication events of a single ancestral *msh* gene, and subsequent divergence early in the vertebrate line, may have given the genes a special role in development of vertebrate-specific structures.

The limb was chosen as a model developmental system in which to analyse patterns of *Msx* gene expression by whole-mount *in situ* hybridisation in mouse and chicken embryos, to relate these patterns to developmental processes known to be occurring and hence to elucidate potential roles for the genes. Expression of *Msx* genes in a number of areas of cell death, proliferation and differentiation suggested that they are involved in a wide range of developmental decisions in several tissues, although their molecular role is still uncertain. The potential for functional redundancy between the genes was addressed. There are distinct differences between the expression patterns of *Msx1* and *Msx2* during limb development; these differences are largely quantitative, however, and the broad similarities between the genes suggest that a large degree of functional redundancy may be possible.

At all stages of limb development the expression of the *Msx* genes and the reported expression of bone morphogenetic protein-4 (BMP4) coincide. By implanting beads, soaked in BMP4, into the chick wing *in ovo*, I showed that BMP4 is an upstream activator of *Msx1* and *Msx2* in the limb. This made it possible to suggest elements of the genetic pathways which may be involved in epithelial-mesenchymal interactions at disparate sites around the body, and to suggest a scenario for the evolution of *Msx* gene expression patterns.

The control of *Msx1* was further investigated using two reporter genes which express β -Gal/*Msx1* chimaeric protein under control of *Msx1* regulatory sequences. Both reporters transcribe *lacZ* from 4.7kb of *Msx1* 5' promoter, but one also contains 7kb of *Msx1* sequence 3' of *lacZ*, including the intron, 3'UTR and 3' genomic sequence.

An assay was developed, by means of which the reporter constructs could be introduced into cells in culture by microinjection, then grafted into the developing chicken limb *in ovo*, using X-Gal staining to visualise expression of the gene. The primary aim was to find sequences in the promoter of *Msx1* which are responsible for induction of *Msx1* expression in response to the signal from the apical ectodermal ridge (AER) of the limb.

10T^{1/2} cells were injected with these constructs; the shorter construct, pH7lacΔ3', was expressed constitutively, whereas the longer one, pH7lacT, was infrequently expressed, showing that there are elements 3' of the transcription start site which had a negative regulatory effect on the expression of *lacZ*. The experiments demonstrated that the 4.7kb is almost certainly not the whole of the *Msx1* promoter; in grafted cells, expression of pH7lacT was not induced in response to the AER. The failure of the AER response could have been due to deficiencies within the promoter or to a failure of the signal transduction pathway within the cells. Radioactive *in situ* hybridisation showed endogenous *Msx1* and *Msx2* were not induced in 10T^{1/2} cells grafted underneath the AER. However, inability to express endogenous *Msx1* need not preclude expression of the introduced promoter, so further work was necessary to determine whether the reporter is capable of responding to the AER.

The behaviour of the 4.7kb *Msx1/lacZ* reporter gene was therefore further investigated in embryonic tissue from several lines of transgenic mouse which contained the gene. Analysing expression of the transgene in these mice confirmed that the 4.7kb was not the whole of the promoter, although it could reproducibly drive an expression pattern similar to elements of expression of *Msx1*. Grafting transgenic limb mesenchyme under the AER of chicken limbs did not induce *lacZ*, providing further evidence that the AER-response element was not in the 4.7kb.

The transgene was strongly expressed in the cells of the ventrolateral dermamyotome of the somites which are fated to form limb muscles. Expression of *Pax3* was previously the only known molecular marker for these cells. Expression of the transgene is retained in a subset of the developing muscles in the anterior of the limb. Hence the transgene provides a molecular marker for these differentiating myoblasts which can be used in experimental situations, and which I used to show that mouse myoblasts can contribute to chicken muscle.

BMP4 could neither induce nor maintain expression of the transgene in cultured limb mesenchyme. The missing AER/BMP4 response element(s) may be one reason why expression of the transgene differs from that of the endogenous *Msx1*, but it is not the only reason. Although transgenic mouse limb tissue is initially responsive to position-specific signals within the chicken which can maintain its expression, it later becomes refractory to induction by these same signals (at stages when the endogenous gene is still responsive), and non-expressing transgenic mesenchyme does not reinitiate expression when grafted into appropriate areas of the chicken or the mouse.

This has the characteristics of an epigenetic silencing effect; it raises the possibility that chromosomal location is important even for regulation of non-clustered homeobox-containing genes, but again suggests that sequences missing from the 4.7kb allow the promoter to fall under the control of repressor proteins which do not affect the endogenous gene.

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CHAPTER ONE

INTRODUCTION

The study of developmental biology:

The study of developmental biology is an attempt to understand the processes which turn a single-celled zygote into a multicellular organism. It is a story of symmetry breaking and pattern formation, resulting in cell movement and differentiation, under the control of the organism's genetic program, often influenced by the environment in which the organism develops.

Approaching the problem of embryogenesis from a genetical point of view, we wish to understand how a series of cascades of gene expression build an organism - the molecular-genetic basis of positional specification and structural morphogenesis.

THE HOMEBOX

A central component of the molecular-genetic mechanisms of embryonic development is a diverse group of proteins which are structurally related by the presence of a homeodomain (McGinnis *et al.*, 1984; Gehring *et al.*, 1990). This is an evolutionarily conserved 61 amino acid motif, which has been shown to bind DNA *in vitro* and *in vivo* (Johnson and Herkowitz, 1985; Odenwald *et al.*, 1989; Otting *et al.*, 1988) with sequence specificity primarily determined by the third and fourth helices of the domain (Treisman *et al.*, 1992; Gehring *et al.*, 1994). Homeodomains from different proteins have different sequences, and hence have different binding specificities for different target sequences on DNA (Desplan *et al.*, 1988; Corsetti *et al.*, 1992; Catron *et al.*, 1993).

Homeodomain-containing proteins can bind sequences in the promoters of other genes and regulate their transcription (Driever *et al.*, 1989; Hayashi and Scott, 1990; Vershon *et al.*, 1995; Catron *et al.*, 1995). Some of the target genes are known (Gould *et al.*, 1990; reviewed in Andrew and Scott, 1992; Jones *et al.*, 1992a; Lawrence and Morata, 1994). All homeobox genes are therefore potential transcription factors.

The homeodomain was originally recognised in the homeotic genes of the *Drosophila* ANT-C and BX-C complexes (hence the name) (McGinnis *et al.*, 1994a,b), but was later discovered in genes not functionally related or physically linked to these complexes. Because homeodomains are found in plant, animal and fungal genes, the motif is at least 1 billion years old (Shepherd *et al.*, 1984; Vollbrecht *et al.*, 1991).

Control, function and evolutionary significance of homeobox-containing genes:

The functions of homeobox-containing genes:

Antp-class genes in vertebrates and invertebrates:

Many metazoan phyla have one or more conserved clusters of homeobox-containing genes, related to a presumed ancestral, HOM-C, complex, whose function has been linked to the developmental program of the animals (McGinnis *et al.*, 1984; Beeman, 1987, 1993; Ueno *et al.*, 1992; Duboule and Dolle, 1989; Wang *et al.*, 1993; Garcia-Fernandez and Holland, 1994).

Different genes of the HOM-C complex have different fields of expression along the anterior/posterior axis of the developing embryo (3' genes are generally expressed more anteriorly) (Akam, 1987; Graham *et al.*, 1989). In *Drosophila*, the combination of genes expressed in the cells of each segment at the cellular blastoderm stage specifies the future development of the segment according to its position along the axis (Akam, 1988; Pankratz and Jackle, 1990; Ingham, 1988; Peifer *et al.*, 1987). Knockout and ectopic expression experiments in mice have suggested that the vertebrate homologues of HOM-C genes (*Hox* genes) may also be acting as segmental selector genes along the rostro-caudal axis of the body (Zhang *et al.*, 1994; Satokata *et al.*, 1995; Rijli *et al.*, 1994) but many of these experiments are interpretable as *Hox* genes controlling growth rates and the timing of proliferation in many somite and neuroectoderm-derived structures (Condie and Cappechi, 1994; Rancourt *et al.*, 1995).

Other classes of homeobox genes:

It would be an error to take the example of the *Antp*-class genes as the archetype of homeobox gene action. There are many more homeobox genes in the other families (reviewed in Duboule, 1994). In vertebrates, homeobox genes of the *Otx*, *Emx*, *Dlx*, *En*, *Pax* and *Nkx* families (reviewed in Puelles and Rubenstein, 1993; Fraser, 1993) have expression domains in the mid- and forebrain which suggest they are acting as homeotic selector genes for segmental (neuromeric) structures (like *Hox* genes caudal to the mid-hindbrain boundary). Many of these genes have a homologue in *Drosophila* (and other invertebrates) which may also be specifying compartments in the head (Cohens and Jorgen, 1990; Hidalgo, 1994). This, together with common elements of expression and control, in invertebrates and vertebrates, of genes such as *en/En*, *tinman/Csx*, *cdl/Cdx*, *prospero/Prox*, *dll/Dlx* and *ey/Pax6* in other areas of the body (Robinson and Mahon, 1994; Gamer and Wright, 1993; He *et al.*, 1989; Quiring *et al.*, 1994; Hadler *et al.*, 1995; McMahon *et al.*, 1992; Davidson *et al.*, 1988; reviewed in Manak and Scott, 1994) suggest there are similarities between the story of the *Antp*-class genes and that of the non-*Antp*-class:

- (i) many of the genes appear to have undergone duplications early in the vertebrate line, leading to families of paralogues with differing expression patterns;
- (ii) there is a tendency for genes to retain aspects of expression domains, and perhaps function, which would have been present in the common ancestors of flies and vertebrates e.g. the *Drosophila eyeless* gene and its vertebrate homologue, *Pax6*, are both expressed during, and are fundamental to, eye development (Osorio and Bacon, 1994; Grindley *et al.*, 1995; Halder *et al.*, 1995).

(Neither (i) nor (ii) are specific to homeobox genes - other developmentally important genes e.g. Wnts, FGFs etc. have undergone duplication steps with similar consequences (Holland, 1994).)

Homeobox gene duplications and the evolution of the vertebrate body plan:

Vertebrates are structurally more complex than their immediate invertebrate ancestors:

The vertebrate body plan is structurally complex and shows features which are definite evolutionary advances over the hemichordates and the non-vertebrate chordates. These vertebrate innovations are pronounced in the head region. They include the mesenchymal neural crest, branchial arches, teeth, paired sense organs, the tripartite brain and the cranial ganglia. In the trunk, there is extensive novel organogenesis, the spinal ganglia, the nephrotome and the sclerotome (Holland, 1992; Gans and Northcutt, 1993).

Increased vertebrate complexity may have resulted from a series of gene duplication events occurring early in the vertebrate line:

Duplication events of homeobox-containing genes early in the vertebrate line have correlated with more widespread expression of the genes (Schugart *et al.*, 1989; Ruddle, 1994). The cephalochordate, *Amphioxus*, expresses the genes of its single *Hox* cluster only in the neural tube (Garcia-Fernandez and Holland, 1994), in contrast to vertebrates, where the genes are expressed in mesodermally derived structures too. Paralogous vertebrate *Hox* genes show different tissue- and domain-specific expression patterns which suggest that they may be doing different, but overlapping jobs, even at the same axial level (Gaunt and Singh, 1990; Gaunt, 1991) - this suggestion is supported by some knockouts of multiple paralogues (Condie and Cappechi, 1993; 1994).

Control of homeobox-containing genes:

Control of expression of homeobox-containing genes is obviously fundamental to their action (since their misexpression during early embryogenesis can cause large changes in the body plan), but is characterised for only a minority, and these incompletely. There are a number of ways of investigating the control of homeobox genes. These include:

- (i) classical genetic analysis;
- (ii) studying misexpression of homeobox genes in animals mutant for a potential upstream gene;
- (iii) causing misexpression experimentally by ectopic misexpression of a potential upstream gene;
- (iv) direct promoter sequencing to identify where control proteins may bind, and immunoassays, DNaseI footprinting or South-western blotting to show that they do;
- (v) modulation of homeobox gene activity by application of cell-extrinsic factors.

The cascade of gap, pair-rule and segment polarity genes which control the initiation of the HOM-C in domains along the a/p axis of *Drosophila* (Akam *et al.*, 1988; Ingham *et al.*, 1988; Peifer *et al.*, 1987)) was inferred mainly from genetic data. In experimental systems, more defined interactions between the promoters of homeobox genes and upstream transcription factors have been described. The zinc-finger gap gene *Kruppel*, *Kr*, upregulates the P1 transcript of *Antp* but downregulates *Ubx* (Ingham, 1988). *hunchback*, *hb*, represses *Ubx* (Ingham, 1988), and binding sites for *Kr* and *hb* have been found in the *Ubx* promoter (Akam, pers.comm.). *hunchback* itself is upregulated by *bicoid*, a homeobox gene (Driever *et al.*, 1989). Hence the homeobox genes can control each other. There are more direct examples, *fushi tarazu*, *ftz*, can upregulate its own expression (Hiromi and Gehring, 1987; Dearolf *et al.*, 1990), as can *Ubx* in the *Drosophila* viscera (Bienz and Tremml, 1988). Ectopically expressing *Deformed* on a heat shock promoter induces and maintains expression of endogenous *Deformed*, also by autoregulation (Kuziora and McGinnis, 1988). Expression of *Abd-A* or *Abd-B* downregulates expression of *Ubx*, which in turn can cause downregulation of *Antp* (reviewed in Hayashi and Scott, 1990). In the vertebrates, *Hoxc3*, *Hoxc4* and *Hoxd4* all upregulate expression of *Hoxc3* (Arcioni *et al.*, 1992).

In *Drosophila*, the Zn-finger gap genes also control expression of pair-rule genes, some of which have homeoboxes. The *even-skipped* promoter has binding sites for *hb* and *Kr*, which activate expression of the third *eve* stripe (Stanjovec *et al.*, 1989). The products of the *hairy* and *runt* genes, as well as *eve* itself, are necessary to maintain *eve* expression subsequently (Goto *et al.*, 1989).

Application of exogenous retinoic acid (RA) affects expression of many, but not all, vertebrate homeobox-containing genes (Izpisua-Belmonte *et al.*, 1991; reviewed in Morris-Kay, 1993). Application

of retinoic acid to mouse embryonal carcinoma, EC, cells induces expression of *Hox* cluster genes. The genes of the 3' end of the clusters are induced at lower RA concentrations than those of the 5' end, and the genes are temporally induced in strict 3'-5' order (Simeone *et al.*, 1990, 1991). Hence temporal and spatial colinearity could be explained by a gradient of RA along the a/p axis, increasing caudally, although there is no evidence that such a gradient exists.

Retinoic acid response elements have been defined in the promoters of several genes (Balling *et al.*, 1989; Marshall *et al.*, 1994); it is possible that retinoic acid receptors bind homeobox gene promoters directly - Simeone *et al.* (1991) showed that activation of some of the *Hox* genes by retinoic acid requires no *de novo* protein synthesis - but in other cases RA may induce secondary signals which indirectly affect expression of the homeobox genes. For example, teratogenic misexpression of *Hoxb2*, induced by retinoic acid (Morris-Kay *et al.*, 1991), may result from misexpression of *Krox20*. The *Hoxb2* promoter has binding sites for *Krox20*, which is normally expressed in rhombomeres 3 and 5 (Wilkinson *et al.*, 1989) and which upregulates *Hoxb2* in these rhombomeres (Sham *et al.*, 1992, 1993) but is ectopically expressed in other rhombomeres in the presence of retinoic acid. Mice which are homozygous null for *Krox20* lose *Hoxb2* expression in rhombomeres 3 and 5 (Swiatek and Gridley, 1993).

In the hindbrain, RA can also affect the expression of other classes of homeobox-containing genes (e.g. *En1* at the mid-hindbrain boundary) and non-homeobox genes (e.g. *Fgf3*), which suggests it may be acting through a series of downstream factors (reviewed in Morris-Kay, 1993). It can also cause misexpression of more rostral homeobox genes (the *Otx* and *Emx* families) (Simeone *et al.*, 1995).

Mutation in the basic-domain leucine zipper gene, *kreisler* (*kr*), also results in defects in hindbrain segmentation and misexpression of *Hoxb* genes, which suggests it is upstream of *Hox* genes (Cordes and Barsch, 1995). The expression of *Krox20* is also affected in *kr* mutant mice, so *kr* may regulate *Hox* genes by regulating *Krox20*.

In the limb, retinoic acid indirectly upregulates expression of *Hoxd11*, *d12* and *d13* via upregulation of the *Shh* gene (see page 37). This example provides an entry to the *in vivo* extrinsic factors which control homeobox gene expression, as there are examples of known cell-signal growth factors which modulate expression of homeobox genes in cells which receive the signal. *Fgf4* can maintain expression of *Evx1* in the distal cells of the vertebrate limb (Niswander and Martin, 1994). The homeobox gene *Xnot* is controlled in the *Xenopus* gastrula by the action of FGF and activin (see page 219). In the *Drosophila* embryo and adult imaginal discs, the secreted product of the *wingless* gene is received by cells adjacent to those secreting it, thus maintaining expression of *engrailed*, possibly via a cell-signalling pathway which involves the gene product of *armadillo* (DiNardo *et al.*, 1988).

Very few systematic sequence studies of vertebrate homeobox gene promoters have been performed. An analysis of the mouse *Msx1* promoter revealed consensus binding sites for Sp1, AP2 and bicoid(!) (Townley, 1995).

The factors which control expression of homeobox genes are thus generally the same as those which control other genes. This is probably true both at the level of immediate transcription factor binding to the promoter and at the level of extrinsic cellular signalling which induces expression, (e.g. *wg*, *hh* and homologues). The clustering of the homeotic HOM-C genes probably has a control significance, and there is no doubt that the control of homeobox-containing genes is very tightly regulated and may be quite complex, but there is no reason to regard the control of the genes as a black box. They are accessible to study at the manipulative experimental level, at the biochemical level and at the genetic level.

Studying the control of different homeobox-containing genes, both within and between species, has already revealed interesting things about the possible evolutionary history of the genes, and may yet reveal more. Control pathways may be conserved - knockouts of the *Drosophila* gene, *posterior sex combs*, *Psc*, (a member of the *Polycomb* group genes which regulate expression of the homeotic genes) and its mouse homologue, *bmi*, both produce posterior homeotic transformations (Van der Lugt *et al.*, 1994). This suggests that the fly and mouse homologues may be controlling the same downstream homeotic gene homologues - this degree of conservation testifies to the fundamental importance of correct control of these genes.

If genetic pathways are truly being conserved across such time-spans, at what point do they diverge to produce different animals?

Perspectives and Questions on homeobox gene activity:

Homeobox-containing genes are transcription factors. They can therefore do many jobs, but seem to have special roles in development, affecting the fate of the cells in which they are expressed. In *Drosophila*, homeobox genes specify segment identity, but there are also maternal effect genes (e.g. *bicoid*), gap genes (*caudal*), pair rule genes (*ftz*) and segment polarity genes (*engrailed*) which contain homeoboxes (reviewed in Duboule, 1994). Hence they can help to specify developmental compartments, certainly in the invertebrates and possibly in vertebrates too (*Nk2* genes, *Otx*, *Emx*, *Dll*). They can act as organiser genes (e.g. *goosecoid* at gastrulation (Blum *et al.*, 1992) and *LIM1* as a mammalian head organiser (Shawlot and Behringer, 1995)). They may be expressed in a tissue specific manner during development

(e.g. *Csx* in myocardium (Manak and Scott, 1994), *Cdx1* in gut endoderm (Gamer and Wright, 1993), or be specific to a cell lineage e.g. *lin-11* - (Freyd *et al.*, 1990), *apterous* specifying subsets of *Drosophila* abdominal muscles and subsets of interneurons (Bourgouin *et al.*, 1992; Lundgren *et al.*, 1995). Some, like the Oct proteins, may be part of the basic transcriptional apparatus (Scholer, 1991). Homeobox genes can affect cell behaviour (*mab-5* influences migration of cells in nematodes (Kenyon, 1986; Costa *et al.*, 1988)), and in yeast, influence the determination of cell mating type (e.g. MAT α 2) (Johnson and Herkowitz, 1985).

The biochemical reason why so many developmentally-important genes contain homeoboxes is not known. There is no intuitive reason why so many selector genes contain homeoboxes, except to say that this is just the way it happened. Plants have homeotic selector genes too, but these have MADS boxes - conserved amino-terminal DNA binding and dimerisation domains (Weigel and Meyerowitz, 1994) not homeoboxes. Mutations in plant homeobox-containing genes can result in aberrant development but these are not really homeotic mutations e.g. *Knotted-1*, which causes mosaic rearrangement of cell types in maize, leading to abnormally roughened leaf sheaves (Vollbrecht *et al.*, 1991).

There may be some special property of homeoboxes which means that genes which contain them can do things that genes without them cannot. To see if this is so, it would be interesting to find out whether there are common links between the control and function of the different classes of homeobox-containing genes during the determination of positional specification and cell fate.

Answering this question probably requires a reassessment of what is meant by the terms 'positional specification' and 'cell fate determination' - since the boundaries between the two are not always clear.

To take a homeotic mutation, such as the inappropriate expression of the *Antennapedia* gene in the antennal imaginal discs which transforms them into legs (Gehring, 1966; Frischer *et al.*, 1986). This implies that there is some stage during the development of the imaginal disc when its future pattern of morphogenesis is undetermined - it can form either a leg or an antenna. It also implies that the imaginal disc is self-sufficient for the patterning information which will control its future development - it has an integrated identity. The disc starts as a clump of cells which have only basic patterning information; once it starts to proliferate and develop, the cells interact with each other in a series of stepwise processes which control and in turn are controlled by a cascade of gene activity which refines (and complicates) the patterns of differentiation occurring in the developing disc.

When the disc starts to develop into either a leg or an antenna, many genes will be expressed. There will certainly be a large degree of overlap between the genes which are used to build a leg and those which build an antenna - the same broad tissue types, using the same structural proteins, are present, but the pattern in which these tissue types are laid out is different. The expression of *Antp* during disc

development (either directly or by means of the action of one or more downstream genes) must modify the kinetics of expression of a secondary rung of 'pattern forming' genes - controlling cell proliferation and cell-cell interactions etc. - and hence modify the whole cascade of gene expression which pushes different cells into different fates in the right places at the right times to form a leg.

In the absence of Antp protein, and in the presence of a number of other HOM-C proteins, the initial kinetics of the cascade of gene expression kick-start the cells into patterns of proliferation, cell signalling interactions and resulting differentiation in the right places at the right times to form an antenna (Struhl, 1981).

A hostile reader would say that this is obvious; it is, but the point it makes is that it is also obvious that the leg/antenna decision is intuitively explicable and that the concept of positional specification by painting of the a/p axis (using HOM-C paint) may not be overly helpful when trying to understand what is really happening.

There is therefore nothing magic about homeosis, and the HOM-C genes need not hold the monopoly on specifying position-appropriate differentiation pathways. Indeed, much of what we regard as gross positional specification may be explained by more subtle effects on cell behaviour which are thoroughly explicable in terms of the activity of several classes of homeobox containing genes, and other classes of non-homeobox genes too.

Expression of *engrailed*, for example, is a marker for cells in the posterior compartment of the leg and wing imaginal discs. As implied by the term 'compartment' it has effects on cell behaviour, as cells expressing *engrailed* are largely prevented from encroaching into the anterior of the wing (Morata and Lawrence, 1975). It is increasingly being recognised that *engrailed* expression probably leads to the expression of cell surface markers, different from those expressed in the anterior compartment and which are incompatible with prolonged existence in the anterior compartment - cells recognise they are not 'with their own kind'. Thus it is that dissociated wing imaginal discs reassociate into clumps of anterior and posterior cells in culture (Garcia-Bellido, 1966; reviewed in Martinez-Arias, 1989). This is reminiscent of cell movement following cuticle rotation in *Dysdercus* (Nubler Jung, 1974), where it looks like cells move until they find neighbours who express the same surface molecules as themselves - the implication being that there is a gradient of cellular affinities across the segment, and that cell proliferation and movement occur to restore the stable array of affinities after experimental manipulation; it also has overtones of the polar co-ordinate model of limb development (French *et al.*, 1976), where cells at a positional discontinuity proliferate until the discontinuity is evened out - it again suggests that a discontinuity of cellular affinities is inductive of proliferation. This would involve recognition of different molecules on

the surface of ones neighbours and proliferation until ones neighbours were almost identical to oneself (when the cell interface is no longer inductive) (reviewed in Martinez-Arias, 1989). Chicken rhombomeres have been shown to comprise lineage-restricted units which may be compartments (Fraser *et al.*, 1990). It is possible that expression of the *Hox* genes specifies the cellular properties which prevent cell mixing.

Expression of the *LIM* class homeobox genes in the developing vertebrate nervous system provides another opportunity to interpret 'positional specification' in terms of defined changes in cell behaviour (reviewed in Lumsden, 1995; Tosney *et al.*, 1995). The motor neurons of the developing CNS are longitudinally arranged into columns, with all the cells in one column sending out axons to the same sets of target muscles. It was shown (Tsuchida *et al.*, 1994) that different columns show different combinations of *LIM* gene expression which 'paint them different colours' in a combinatorial manner, mirroring that of the *Hox* genes along the A/P axis. It could be said that the *LIM* code specifies the targets to which the axons of any one column migrate. It is known that motor neuron axons migrate towards cues, possibly chemoattractant molecules, which guide them to their targets, with axons from different columns responding to different chemoattractants (or repellents), guiding them to different targets (Lance-Jones and Landmesser, 1981; Tosney, 1992; Ferguson, 1983; Dodd and Schuchardt, 1995). The *LIM* genes are therefore good candidate determinants of the nature of the signals to which the migrating axons respond, especially as their expression is downregulated once the target is innervated. They may, either directly or via their downstream genes, change the expression of cell surface receptors for different guiding molecules, or alter aspects of the signal transduction pathway between the receptor, the nucleus and the expression of cytoskeletal and adhesion molecule proteins (affecting migration). It is clear however that to regard *LIM* genes as 'painting' columns with 'positional specification' is an unnecessary abstraction.

Elucidation of the control of development by homeobox-containing genes of any class will come when anthropomorphic terms such as 'positional specification' are defined in terms of cascades of gene activity affecting cell lineages, the potential to collaborate in cell-cell interactions, cell movements and the potential to differentiate. The link between homeobox gene expression and the response to extrinsic signals is becoming more explicit all the time - Grueneberg *et al.* (1995) showed that the *Phox* gene guides serum response factor, SRF, to its DNA binding site, SRE, and hence potentiates the cellular response to growth factors in the serum.

A lot of work has been done on homeobox-containing genes and it is likely that they are involved at all stages of the stepwise processes which lead to the determination of cell type. There are still many questions, not least of which is to ask what each individual homeobox gene does, at the molecular level, during embryonic development. In a more global sense, we want to know what the homeobox genes are doing as a family. To take the definition of molecular homology to its extreme, there must originally have been one primordial homeobox gene, from which all the others are descended. Can we see the shadow of the ancestral homeobox gene in its descendants? What aspects, if any, of the control and function of homeobox genes are common to all? What has changed between the classes? How has the evolution of the classes of homeobox genes contributed to patterns of morphological change? Only a comprehensive study of all classes of homeobox gene activity will answer these questions.

Proteins which contain homeodomains are interesting. The developmental biologist is interested in discovering what different homeodomain-containing proteins are doing, but as an experimental biologist, needs an accessible system in which to work. The homeodomain itself is not beautiful; the developing embryo is, and the *sine qua non* of studying homeobox genes is that we want to be able to look at a developing embryo and understand what is happening to it. It is now true to say (Saunders, 1948; Saunders Jnr., 1966) that generations of biologists have found the vertebrate limb to be 'their' developmental system in which to ask questions. How does the limb develop? What genes are used and what does this imply for development of the rest of the body, and animal development in general?

Although the molecular pathways in which homeobox genes and growth factors operate may have been conserved, the vertebrate limb is a structure not present in our immediate non-vertebrate ancestors. Genes which control the formation of this structure are therefore doing a vertebrate-specific job, not only in the limb, but maybe also in other vertebrate-specific additions such as the face, skull and forebrain. Limb and craniofacial defects often occur together in the same animal (Winter, 1994, describes a number of syndromes in which preaxial limb defects are associated with midline craniofacial defects). The pentadactyl limb, as a model developmental system, provides an opportunity to get a handle on what these genes have been enlisted to do. If we understand the development and evolution of the pentadactyl limb, we may find that we also understand the development and evolution of many other parts of the vertebrate body as well.

DEVELOPMENT AND EVOLUTION OF THE PENTADACTYL LIMB:

Introduction:

Vertebrate limbs develop according to a basic plan which has remained recognisably the same in amphibians, reptiles, birds and mammals. In this mould, a variable number of digits (characteristically five) articulate with a variable number of carpal (wrist) or tarsal (ankle) bones. Proximal to this are the 'long bones' which meet at an elbow joint. A bony girdle attaches the limb to the vertebral column, providing a firm fulcrum for the leverage by means of which the legs can propel the body. The bony skeleton is attached by connective tissue to muscles, which, under nervous control, move the limb. The system is vascularised and covered with a layer of skin. In its basic, unspecialised form, the pentadactyl limb serves to hold the body off the ground during locomotion.

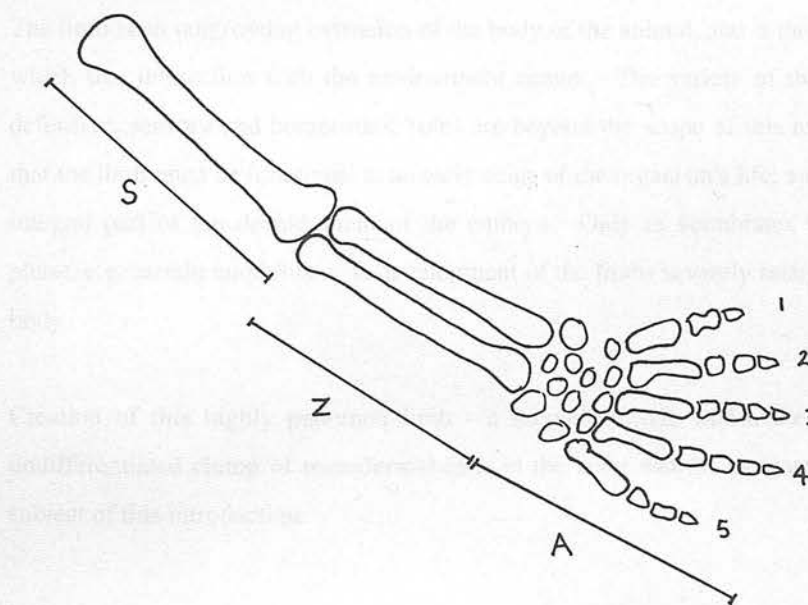


Figure 1.1: Skeletal elements of the unspecialised pentadactyl limb. One proximal long bone (humerus/femur) in the stylopod (S) articulates distally with two zeugopodal (Z) long bones (radius/tibia anteriorly and ulna/fibula posteriorly). The complex autopod (A) contains variable numbers of small carpal/tarsal elements, articulating with longer metacarpals/metatarsals and hence with 5 digits, each with a number of phalange bones. Digit 1 (the thumb or big toe in humans) is the most anterior. Along the third, dorso-ventral axis, 'ventral' corresponds to the palm of the hand (sole of foot).

The shapes of the elements of the limb may vary according to the main tasks that it performs, resulting from the action of classical natural selection. Thus, (although some would say that this is nothing but a series of 'Just So' stories) we see elongation of the digits of bats, supporting the wing membrane for flight, broadening of the humerus in European moles, giving leverage to the 'digging' muscles, extension of the long bones of horses and frogs, providing speed of movement, extension of the pisiform giving pandas a false thumb etc. etc. (Reviewed in Tabin, 1992) Reduction and fusion of the limb elements from the ancestral form is common - birds have only three digits in the wing (and really only use two), horses walk only on their middle toe, (digit 3) which has fused with the rudimentary digits 2 and 4. Ontogeny in the case of bone loss need not recapitulate phylogeny - some bones chondrify but then regress during development, some do not chondrify before regressing and some bones are lost without even an embryonic condensation to show that they were present ancestrally (Shubin and Alberch, 1986).

Conversely, one of the earliest amphibians, *Ichthyostega*, had seven digits. In some animals the digits may not be separated, forming the paddles or flippers of some aquatic mammals. Limbs have been lost completely in some animals which have reverted to an anguilliform method of locomotion (snakes, most famously) and in some cases, such as the human arm, have lost their propulsive function and are used to manipulate the environment with which the organism interacts.

The limb is an outgrowing extension of the body of the animal, and is therefore one of the front lines over which this interaction with the environment occurs. The variety of the adaptations of the skin, in its defensive, sensory and homeostatic roles are beyond the scope of this introduction; it is a fact however that the limb must be functional at an early stage of the organism's life, and development of the limbs is an integral part of the development of the embryo. Only in vertebrates which have an extended 'larval' phase, e.g. certain amphibians, is development of the limbs severely retarded behind that of the rest of the body.

Creation of this highly patterned limb - a secondary axis within the developing embryo - from an undifferentiated clump of mesodermal cells in the body wall of the gastrulated embryo, forms the main subject of this introduction.

Phylogenetic origins of the limb:

There is no palaeontological evidence that the earliest chordates (those found in the 500 million year old Burgess Shale) had limbs (Conway-Morris, pers. comm.). Modern day Cephalochordates and Urochordates lack them, as do Hemichordates. The earliest tetrapod amphibians had limbs, however, and it is the fish to which we must look to find the origins and homologies of the structure.

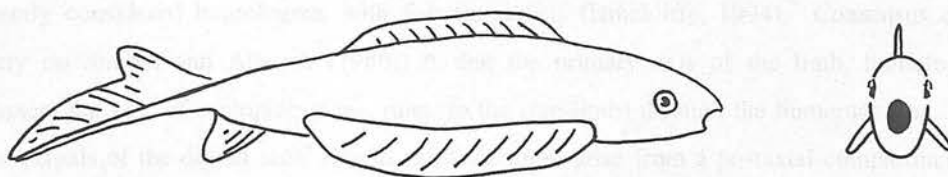
The fish class is subdivided into the 'primitive' jawless fish (e.g. the cyclostomes) and the jawed fish. This latter group may be divided into the cartilaginous sharks and rays, and the bony fish. Although the origins and homologies of the tetrapod limb are much disputed, there is common agreement that it has arisen from the fins of the bony fishes (in Pough *et al.*, 1989).

Fish fins are supported by an internal skeleton to which the muscles of articulation are linked, and the fin itself is supported by an array of dermal bones - the fin rays. Bony fish can be subdivided into the actinopterygians (ray-finned) and the sarcopterygians (lobe-finned). In actinopterygians the dermal rays are the only skeletal elements which commonly stick out from the body wall; the endochondrial skeletal elements are much reduced. In the sarcopterygians however, the endochondrial skeleton bulges out from the body wall, forming the lobe, and this is thought to be the forerunner of the tetrapod limb (in Pough *et al.*, 1989).

For both sarcopterygians and actinopterygians, the primary function of the fin is upthrust and propulsion in the aquatic medium; in the extant lungfish (and extinct vertebrate ancestors), dragging themselves between drying streams, the fin is used to pull themselves over land. The primary adaptations of the fin in early tetrapod evolution adapted the internal skeleton and provided a firm anchorage to the axial skeleton, with the result that the limb could support the weight of the body and provide propulsion on land.

Paired fins possibly evolved by adaptation of a metapleural fin fold, running the length of the flank, found primitively in some Cephalochordates and some extinct taxa. Possibly the development of the fold became restricted to the pelvic and pectoral regions (Tabin, 1992). Evolution of pectoral fins preceded that of pelvic fins, and both were preceded by elaborate medial fins (e.g. dorsal and anal fins) (Coates, 1994) (Figure 1.2).

(a)



(b)

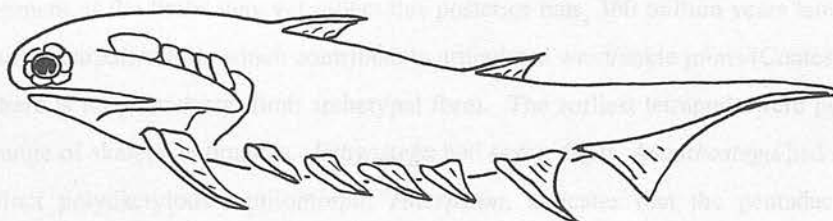


Figure 1.2: Pectoral and pelvic fins may have arisen from subdivisions of a lateral fin fold like that of the extinct jawless Anapsid, *Endeilopis* (a). Alternatively, the ancestral form may have resembled the acanthodian, *Climatius* (b), with many lateral paired fins, most of which were subsequently lost. Adapted from Hinchliffe and Johnson (1980) and Pough *et al.* (1990).

The fin rays, the dermal component of the fin skeleton, are first laid down as proteinaceous rays (dermatrichia), upon which the bony rays (lepidotrichia), of presumed neural crest origin, are lain (Coates, 1994). The transition from a ray-fin to a lobe-fin was presumably achieved by increasing the initial outbudding of the mesoderm at the expense of development of the ectodermal fin fold in which the dermal rays are formed (Hinchliffe and Johnson, 1980).

In living families of sarcopterygians, the latimerians (Coelacanth) and the dipnoans (lungfish), it is possible to see affinities between the skeleton of the lobe and the skeleton of the tetrapod limb. There is however one extinct family (examples of which have been found in Scottish deposits 360 million years old), called the Osteolepiforms (a subfamily of the sarcopteryginous Crossopterygians), where the homologies of the long bones with those of tetrapods are more obvious. Osteolepiforms, on the basis of their limbs, their labyrinthodont teeth and certain forgettable features of the skull, are thought to be the direct ancestors of the tetrapod line (in Pough *et al.*, 1989).

While the Osteolepiform limb certainly has bones which could become the carpals and digits of its evolutionary descendants (Figure 1.3), it is proving impossible to get agreement about the specific homologies between these distal bones and the distal bones of the tetrapod limb. Tetrapod digits are not presently considered homologous with fish fin radials (Hinchliffe, 1994). Consensus opinion (based largely on Shubin and Alberch (1986)) is that the primary axis of the limb, homologous with the metapterygial axis of sarcopterygians, runs (in the fore-limb) through the humerus, ulna, ulnare and the distal carpals of the digital arch. Digits must therefore arise from a postaxial compartment of the limb, and may be neomorphic (Duboule, 1994). (Later in this chapter I will describe evidence that the development of the limbs may yet reflect this posterior bias, 360 million years later). Digits arose before the ossified carpals/tarsals which contribute to articulated wrist/ankle joints (Coates, 1994).

There is no pentadactyl limb archetypal form. The earliest tetrapods were polydactylous and had a large range of skeletal anomalies. *Ichthyostega* had seven digits, *Acanthostega* had eight. The presence of an extinct polydactylous reptilomorph, *Tulerpeton*, indicates that the pentadactylous form stabilised independently in the amniotes and the amphibians (Coates, 1994).

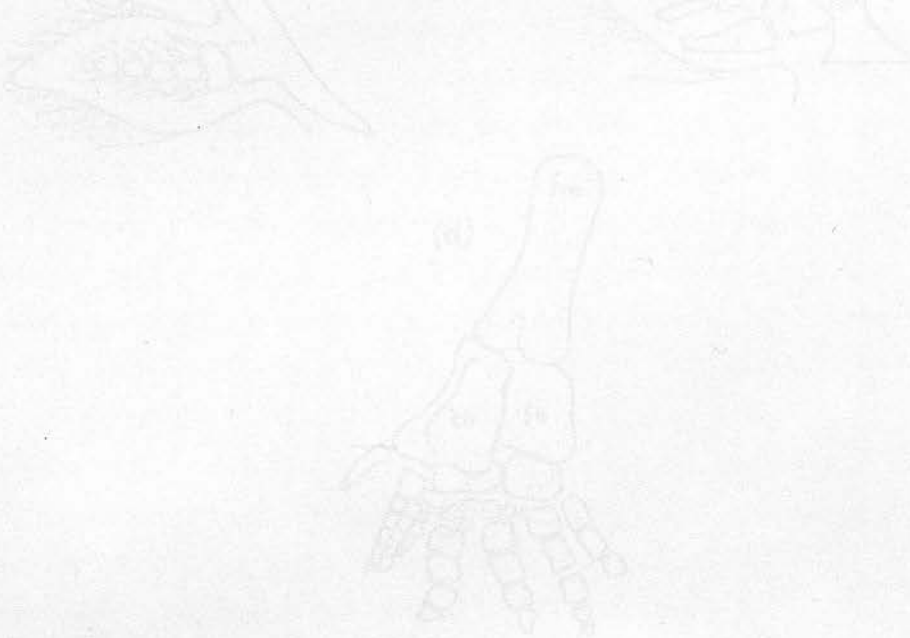
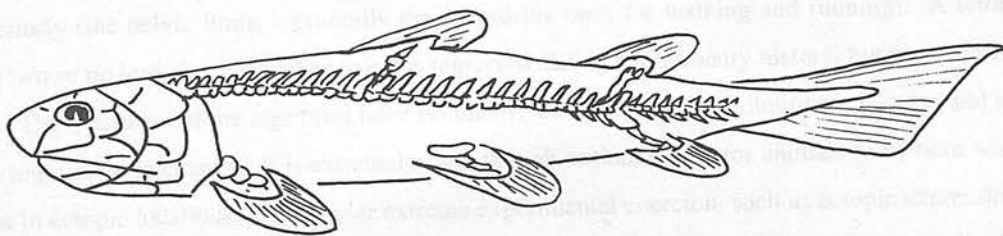
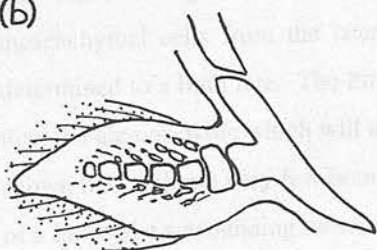


Figure 1.3: The skeletal elements of a tetrapod limb. (a) Lateral view of the limb, showing the humerus, ulna, radius, and the distal carpals. (b) Medial view of the limb, showing the humerus, ulna, radius, and the distal carpals. The diagram illustrates the homology between the bones of the tetrapod limb and the bones of the fish fin.

(a)



(b)



(c)



(d)

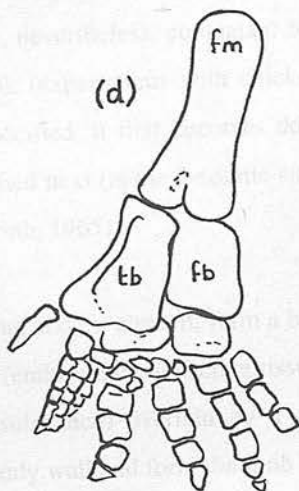


Figure 1.3: (a) *Osteolepis* - a potential tetrapod ancestor

(b) A modern Dipnoan (lungfish) fin, showing skeletal elements

(c) Skeletal elements of an osteolepiform fin

(d) Skeletal elements of the limb of *Ichthyostega*

Abbreviations: sc-cor - scapula-coracoid; h - humerus; r - radius; u - ulna; fm - femur; fb - fibula; tb - tibia.

Adapted from Pough *et al.*, 1990; Coates, 1994

Ontogenetic origins of the limb:

Tetrapods have a pair of limbs at the anterior of the trunk (the pectoral limbs, arms or wings) and two posteriorly (the pelvic limbs - generally the propulsive ones for walking and running). A tetrapod may have two or no legs if one or either pair has regressed during evolutionary history, but none has more than four. The positions of the legs must have become fixed very early in evolutionary history, and seem well nigh impossible to change. It is extremely rare, though not unknown, for animals to be born with normal limbs in ectopic locations; even under extreme experimental coercion, such as ectopic expression of such genes as *Hoxb8* or *Fgf4* in transgenic mice, only abnormal limb buds can be initiated from the body wall at sites where they would not normally occur (Charite *et al.*, 1994).

The limbs emerge from four sites in the Wolffian ridge along the flank of the embryo, where mesenchymal cells from the lateral plate mesoderm, together with their overlying ectoderm, become determined to a limb fate. The limb field, the area of tissue which is competent to form a limb, is greater than the area of tissue which will actually do so. Experiments with amphibians (e.g. Harrison, 1918) have shown that within a very few hours of the formation of the limb field, excision of these cells leads to loss of a limb - the surrounding mesenchyme cannot compensate even though the wound heals well. The areas which do not form a limb are, nevertheless, competent to support the development of an ectopic limb which is grafted onto the flank (experiments with chickens and urodeles). Whereas the limb field is initially developmentally unspecified, it first becomes determined to form either an arm or a leg; the anterior-posterior axis is specified next (at the 5-somite stage in chickens), and the dorso-ventral axis last (at the 13-somite stage) (Amprino, 1965).

Cells in the limb field proliferate and, as a result, form a bulge in the body wall. This bulge, consisting of undifferentiated mesenchyme (embryonic connective tissue cells in a loose matrix of collagen fibres and mucopolysaccharide ground substance) overlain by a one-cell thick layer of ectodermal epithelium, continues to grow out of the body wall and form the limb bud.

Because the limb projects from the body wall, it is accessible to experimental manipulation - a model developmental system that forms, from an undifferentiated bud, a simple, well-defined, mainly two dimensional, patterned limb, which is accessible to the biologist and whose function is not fundamental to the future development of the embryo. A great number of non-molecular classical embryological studies have been performed on it since the turn of the century. This work concentrated on bird and amphibian embryos which, unlike mammals, are easily accessible to the investigator.

These manipulations have defined subregions in the limb bud which have different properties and functions.

The Progress Zone:

Cells at the distal margin of the limb bud have a higher proliferation rate than those located more proximally (Hornbruch and Wolpert, 1970). It is the division and growth of these cells which is primarily responsible for outgrowth of the limb bud in the proximo-distal direction (although there is a contribution from forward migration of more proximal cells (Ede and Agerbak, 1968)) and, hence, this group of cells is called the Progress Zone (PZ). Cells which stray from the very distal tip of the limb undergo a drop in proliferation rate and may begin to differentiate (Summerbell *et al.*, 1973). Hence the structural elements of the limb are laid down in a proximal-distal sequence - proximal elements such as the long bones being specified before more distal elements such as the digits.

Experimental embryology was able to propose a model where the proximo-distal specification of a cell was determined by the amount of time it spent in the Progress Zone before differentiating; this model proposes that a molecular clock starts ticking when the limb bud starts to grow, by which cells measure how long they have been in the Progress Zone. Cells which have been in the Progress Zone for only a short time may contribute to a humerus, whereas those that remain there for longer 'know' to contribute to e.g. a digit.

Evidence that a clock exists (and is not just a result of cells which fall out of the Progress Zone later being given a proximo-distal specification by means of morphogen) came from experiments whereby a Progress Zone was explanted from a chicken limb and replaced with a Progress Zone from a younger donor. The donor's Progress Zone continues to develop a series of proximo/distal elements according to its own developmental age, not that of the host, leading to duplication of elements in the proximo-distal direction (Summerbell *et al.*, 1973). Limb elements are missing if the Progress Zone of a younger limb is replaced with that of an older.

The elements of the limb which differentiate from the remaining host tissue are unaffected by the presence of the new Progress Zone, showing that there is no proximo-distal morphogen released by the donor Progress Zone.

Once all the limb pattern elements have been specified - i.e. cells for the distal tips of the digits have been determined - the Progress Zone in its classical sense no longer exists (although the stem cells of the nail

plate which contribute to claws in adult life may represent the last halloo of this tissue). Growth in the limb (which at this time is much smaller than it will be at the end of development) is continued both by general mitotic events in the differentiating tissue and at specific growth points e.g. mitotic hotspots in the bones.

The Apical Ectodermal Ridge:

The definition of the Progress Zone requires that cells know they are at the very distal tip of the limb bud (and maintain their undifferentiated, highly mitotic state) and they know when they have fallen behind and can start to differentiate. Classical embryological manipulations have shown that there is a diffusible signal produced by the most distal ectoderm of the limb bud which prevents differentiation or death and maintains mitosis in the Progress Zone. When cells fall out of the domain of influence of this distal ectoderm, they can begin to differentiate.

This distal ectoderm is, in mammals and birds but not in urodeles, organised into a distinct morphological structure known as the Apical Ectodermal Ridge, (AER) (described in Wanek *et al.*, 1989). This is a ridge of pseudostratified columnar epithelium, 1-3 cells wide, over the very distal tip of the limb in an anterior to posterior direction. It is continuous with the simple cuboidal epithelium which covers the rest of the early limb bud, and from which it becomes morphologically distinguishable during early bud outgrowth. (Fish have a pseudo-AER formed by a folding of the epithelium of the distal tip of the limb.)

The AER can be neither induced nor maintained in the absence of Progress Zone mesenchyme and conversely, the mitotic activity of the Progress Zone is not maintained in the absence of the AER, either after surgical removal or in mutants where the AER is not induced (Singh *et al.*, 1991; Summerbell *et al.*, 1973)(Figure 1.4). Flank mesenchyme cannot maintain an AER. Chicken limb mesenchyme loses the ability to induce an AER after its appearance at stage 18, but acquires the property of ridge maintenance (in Hinchliffe and Johnson, 1980).

The ridge is permissive, not instructive, for limb specification. It probably produces a diffusible signal - it can maintain Progress Zone cells in culture if held apart from them by a permeable Millipore filter of 0.45µm pore-size or bigger (i.e. quite a big protein) (in Hinchliffe and Johnson, 1980).

The AER is also necessary for the maintenance of an irregularly defined domain of cells around the posterior distal margin of the limb and known as the Zone of Polarising Activity (ZPA) (Thakle *et al.*, 1975). The ZPA is positioned along the posterior margin of the limb bud and is the anterior-posterior axis of the limb, possibly as a result of an expansion which forms a concentration gradient along the anterior-posterior axis of the limb bud.

The experimental study for polarising activity is an experiment whereby the ZPA is removed from a chick limb and replaced into the anterior-distal margin of a host limb bud. The limb bud is then exposed to the host limb bud development and a mirror-image duplication is observed with the anterior margin of the limb bud.

The diagram shows the level of limb truncation in the chick after removal of the AER at the stages shown. The diagram shows the level of limb truncation in the chick after removal of the AER at the stages shown. The diagram shows the level of limb truncation in the chick after removal of the AER at the stages shown.

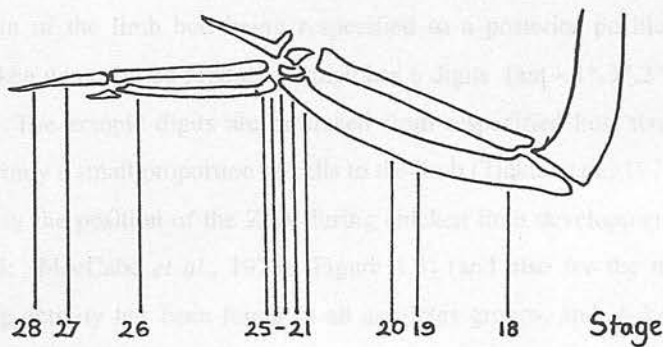


Figure 1.4: From Summerbell *et al.*, 1973. Diagram showing level of limb truncation in the chick after removal of the AER at the stages shown.

Figure 1.4: From Summerbell *et al.*, 1973. Diagram showing level of limb truncation in the chick after removal of the AER at the stages shown. The diagram shows the level of limb truncation in the chick after removal of the AER at the stages shown. The diagram shows the level of limb truncation in the chick after removal of the AER at the stages shown.

ZPA cells can be removed by the posterior-distal margin of the limb bud as a polarising activity, but after removal of the AER, the limb bud is morphologically indistinguishable from the control limb bud. It is therefore concluded that the ZPA has a role in the development of the limb bud and both the anterior-posterior and posterior-distal axes of the limb have been specified.

The AER is necessary for the maintenance of proliferation and activity of the ZPA. In the absence of the AER, ZPA activity is lost and the posterior-distal margin of the limb bud is lost. The ZPA must be removed from the limb bud to be due to the anterior-distal margin of the limb bud. The ZPA is a polarising activity and is necessary for the development of the limb bud. The ZPA is a polarising activity and is necessary for the development of the limb bud.

The Zone of Polarising Activity:

The AER is also necessary for the maintenance of an empirically defined domain of cells around the proximo-distal corner of the limb bud known as the Zone of Polarising Activity, (ZPA) (Tickle *et al.*, 1975). The ZPA imposes positional specification upon cells across the anterior-posterior axis of the limb, possibly by release of a morphogen which forms a concentration gradient along the anterior-posterior axis of the limb, highest posteriorly.

The empirical assay for polarising activity is an experiment whereby the ZPA is removed from a donor limb and implanted into the *anterior*-distal margin of a host limb bud. The famous result of this experiment is that the host limb bud develops ectopic digits in a mirror-image duplication consistent with the anterior margin of the limb bud being respecified to a posterior positional value. In the classic experiment a chicken wing can be produced which has 6 digits (ant - 4*,3*,2*,2,3,4 - post; '*'represents an ectopic digit). The ectopic digits are produced from respecified host tissue, not from donor tissue which contributes only a small proportion of cells to the limb (Tickle *et al.*, 1975).

Using this assay the position of the ZPA during chicken limb development was mapped (Honig and Summerbell, 1985; MacCabe *et al.*, 1973) (Figure 1.5) (and also for the mouse (Wanek and Briant, 1992)). Polarizing activity has been found in all amniotes groups, and in *Xenopus* also (Cameron and Fallon, 1977). There is polarising activity very early in the posterior of the developing limb. Indeed, it may be induced very early in the limb field - experiments with salamander embryos have shown that it is possible to excise the limb field cells, turn them round so that the a/p axis is reversed, and get a limb which is essentially normal, but which points backwards. This implies that the limb, as a secondary axis, is self-sufficient for the pattern forming signals which it requires to undergo morphogenesis, and is largely independent of the development of the primary axis (Hinchliffe and Johnson, 1980).

ZPA cells can be mapped to the posterior/not-quite-distal corner of the limb bud as it grows distally, but after specification of the handplate, before digits are morphologically distinguishable, polarising activity is lost (MacCabe *et al.*, 1973) - by this time the ZPA has done its job and both the anterior-posterior and proximo-distal axes of the limb have been specified.

The AER is necessary for the maintenance of proliferation and activity of the ZPA - in the absence of the AER, ZPA activity is lost, and in the surgical procedures described earlier, the ZPA must be placed next to or close to the anterior AER to be able to respecify the anterior mesenchyme (and will only respecify mesenchyme which lies distally to it - Ispizua-Belmonte *et al.*, 1992). The ZPA indirectly maintains the AER by maintaining cell proliferation in the Progress Zone (see later). Diffusible signals are thought to

be responsible for these effects - thus the AER and the ZPA together maintain the Progress Zone, and can even induce an ectopic one in experimental situations (see later).

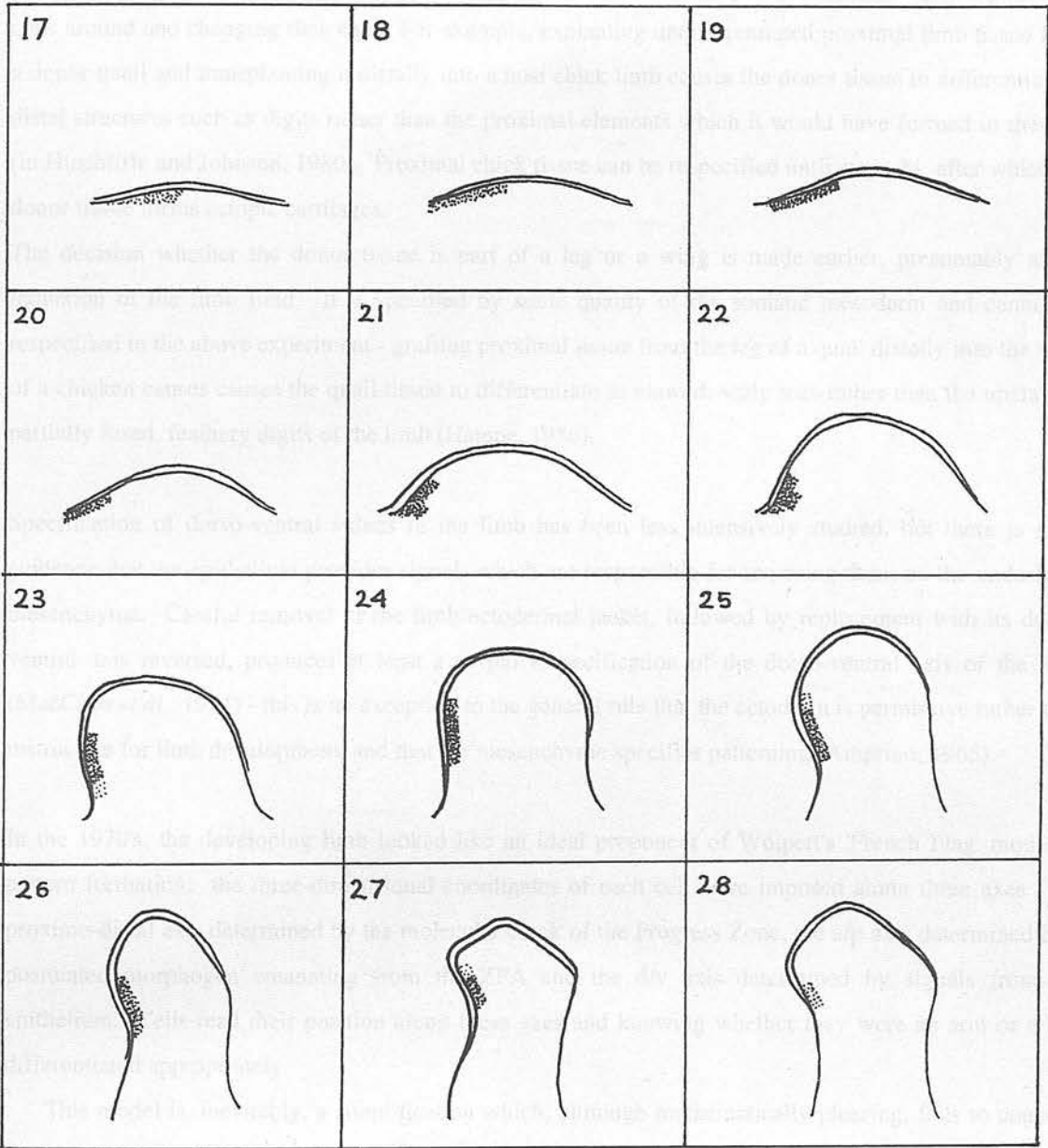


Figure 1.5: The Zone of Polarising Activity (ZPA) (stippling) during chick limb development. Adapted from Honig and Summerbell (1985) and MacCabe and Gasseling (1973). Stages numbered in top left corner. Scale changes between stages. Anterior to the right.

A framework of limb positional specification:

Thus, before any molecular biology had been done on the limb at all, embryological studies had provided a framework by which cells were given positional values along the antero-posterior and the proximo-distal axes. The cells then differentiated appropriately according to their position. These positional values could be respecified - either, as seen above, by ZPA grafts or by moving non-polarising cells around and changing their fate. For example, explanting undifferentiated proximal limb tissue from a donor quail and transplanting it distally into a host chick limb causes the donor tissue to differentiate as distal structures such as digits rather than the proximal elements which it would have formed in the host (in Hinchliffe and Johnson, 1980). Proximal chick tissue can be respecified until stage 24, after which the donor tissue forms ectopic cartilages.

The decision whether the donor tissue is part of a leg or a wing is made earlier, presumably at the induction of the limb field. It is specified by some quality of the somatic mesoderm and cannot be respecified in the above experiment - grafting proximal tissue from the leg of a quail distally into the wing of a chicken causes the quail tissue to differentiate as clawed, scaly toes rather than the unclawed, partially fused, feathery digits of the limb (Hampe, 1956).

Specification of dorso-ventral values in the limb has been less intensively studied, but there is good evidence that the epithelium provides signals which are responsible for imposing them on the underlying mesenchyme. Careful removal of the limb ectodermal jacket, followed by replacement with its dorso-ventral axis reversed, produces at least a partial respecification of the dorso-ventral axis of the limb (MacCabe *et al.*, 1974) - this is an exception to the general rule that the ectoderm is permissive rather than instructive for limb development, and that the mesenchyme specifies patterning (Amprino, 1965).

In the 1970's, the developing limb looked like an ideal proponent of Wolpert's 'French Flag' model of pattern formation; the three-dimensional coordinates of each cell were imposed along three axes - the proximo-distal axis determined by the molecular clock of the Progress Zone, the a/p axis determined by a postulated morphogen emanating from the ZPA and the d/v axis determined by signals from the epithelium. Cells read their position along these axes and knowing whether they were an arm or a leg, differentiated appropriately.

This model is, inevitably, a simplification which, although mathematically pleasing, fails to consider the local cell-cell interactions and cell movements which are overlain upon global specification acts (Shubin and Alberch, 1986). Certainly, it would be an error to regard these three axes as being independent of each other; studies of the patterns of growth of chick limbs (Bowen *et al.*, 1989) have

shown that the a/p and prox/distal axes are neither straight, perpendicular nor invariant with time. Nevertheless, the model remains a framework upon which one can base a series of experiments to investigate pattern formation in the limb.

Stripping down from the biology of the limb to the mathematics of the model is a process that is necessary to capture the essence of the problem. The model is a simplified representation of the limb, and it is important to be aware of the limitations of the model. This is not to say that the model is wrong, but rather that it is a simplification of the real world. The model is a tool to help us understand the underlying principles of the system, and it is important to use it in a way that is consistent with the data.

Cell Death

The most serious form of differentiation is cell death. Cell death is a process that is essential for the development of the organism, and it is a process that is regulated by a variety of factors. Cell death is a process that is essential for the development of the organism, and it is a process that is regulated by a variety of factors.

Cell death is an integral part of the development of the organism, and it is a process that is regulated by a variety of factors. Cell death is a process that is essential for the development of the organism, and it is a process that is regulated by a variety of factors. Cell death is a process that is essential for the development of the organism, and it is a process that is regulated by a variety of factors. Cell death is a process that is essential for the development of the organism, and it is a process that is regulated by a variety of factors.

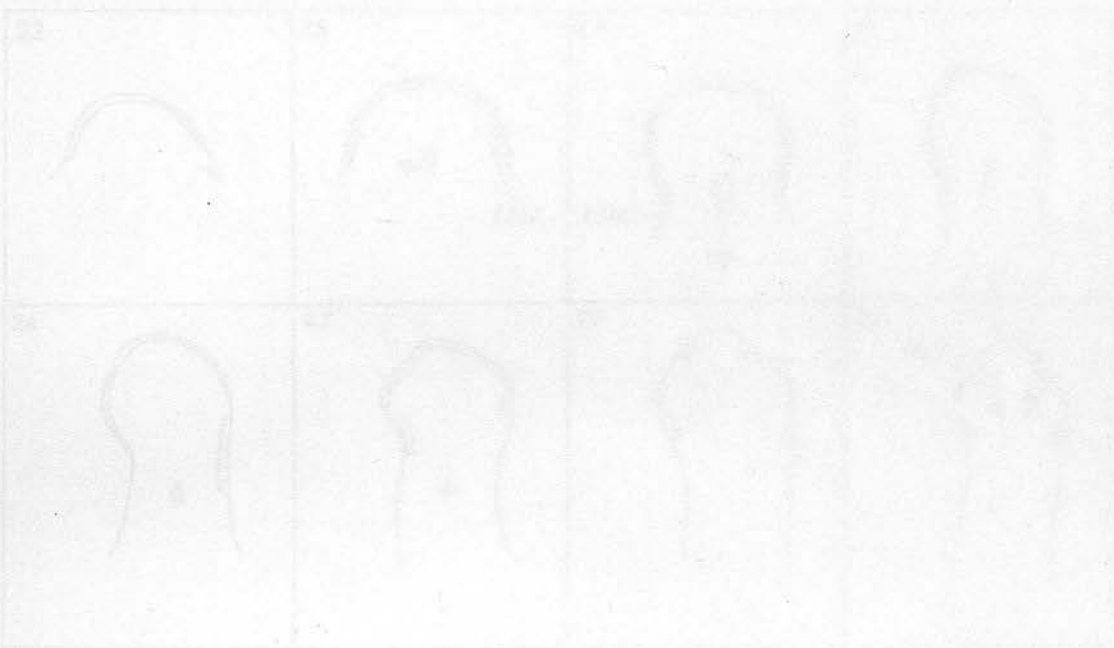


Figure 1.6: Cell death in the developing limb. The diagrams show the progression of cell death in the developing limb, from a single cell to a cluster of cells. The diagrams are labeled with numbers 1 through 8, indicating the sequence of events. Adapted from [reference].

Differentiation processes:

Stepping down from the concept of pattern formation to the hard work of producing a functional limb, it is necessary to consider the processes involved in transforming a limb bud full of undifferentiated (albeit positionally specified!) mesenchyme into a limb with bones, muscle, skin, nerves, connective tissue and blood. This introduction would be incomplete without at least a basic treatment of the physical processes of the differentiation of these tissues.

Cell Death:

The most terminal form of differentiation is apoptotic cell death; tissue modelling by apoptosis is a significant factor in limb development.

Cell death is an integral part of the developmental program of (probably) virtually all multicellular organisms - the dogma being that it removes cells which are unnecessary for the future development of the organism. There are areas of massive cell death in the limb, which were investigated in embryonic chicken wings by Saunders Jnr. *et al.* (1962). These areas of cell death during development were accurately mapped (Figure 1.6).

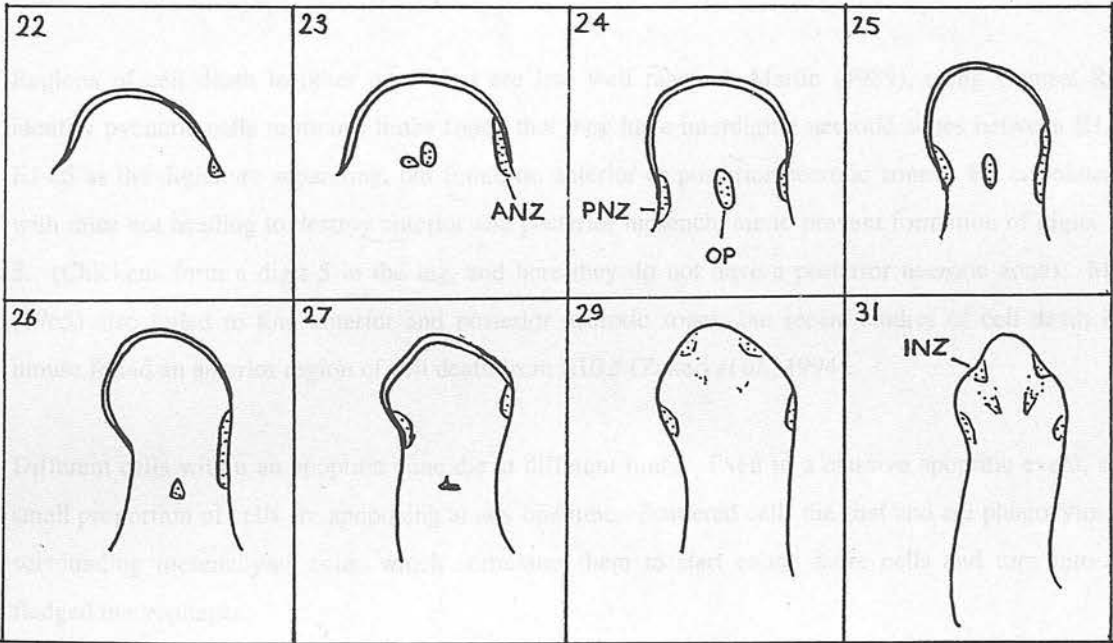


Figure 1.6: Cell death in the fore-limb of chicken from stages 22 to 31. Anterior is to the right.
Abbreviations: ANZ - anterior necrotic zone; PNZ - posterior necrotic zone; INZ - interdigital necrotic zone; OP - opaque patch.
Adapted from Saunders *et al.* (1962) and Hinchliffe and Johnson (1980)

The areas of cell death were christened 'necrotic zones', but are now recognised as regions of apoptosis - programmed, deliberate, cell death in physiological conditions which could otherwise support the cells.

Saunders Jnr. *et al.* defined the following regions of cell death which are associated with structuring of the shoulder, the elbow and wrist, and in defining the digits

- (i) Stages 21 to 23; A proximal anterior region of cell death associated with the sculpturing of the shoulder region.
- (ii) Stages 23 to 30; A region of cell death starting proximally and moving up the anterior margin of the mesenchyme, reaching the proximal anterior boundary of digit 2. This is the anterior necrotic zone - ANZ.
- (iii) Stages 24 to 30; A posterior region of cell death starting proximally and moving relatively more distally to reach the posterior edge of digit 4 - the posterior necrotic zone, PNZ.
- (iv) Stages 31 to 33; Interdigital mesenchymal cell death which causes collapse of the overlying ectoderm and hence separation of the digits. Called the interdigital necrotic zone, INZ. Chickens require the remains of a functional AER to initiate interdigital cell death.

They also identified other regions of less massive cell death in the joints and between the two bones of the forearm (long known to chick embryologists as the 'opaque patch').

Regions of cell death in other organisms are less well mapped. Martin (1989), using Neutral Red to identify pycnotic cells in mouse limbs found that they have interdigital necrotic zones between E13 and E14.5 as the digits are separating, but found no anterior or posterior necrotic zones. He correlated this with mice not needing to destroy anterior and posterior mesenchyme to prevent formation of digits 1 and 5. (Chickens form a digit 5 in the leg, and here they do not have a posterior necrotic zone). Milaire (1965) also failed to find anterior and posterior necrotic zones, but recent studies of cell death in the mouse found an anterior region of cell death from E10.5 (Zakeri *et al.*, 1994).

Different cells within an apoptotic zone die at different times. Even in a massive apoptotic event, only a small proportion of cells are apoptosing at any one time. Scattered cells die first and are phagocytosed by surrounding mesenchyme cells, which stimulates them to start eating more cells and turn into fully-fledged macrophages.

Saunders Jnr. (1962, 1966) performed experiments on the chicken PNZ to investigate the nature of the signals which controlled the position and time of death.

By injecting carbon powder into developing wing buds, he could map out cells in the posterior proximal wing bud of embryos from stage 17 onwards which would form the PNZ at stage 24. Cells became determined to an apoptotic fate very early, since if the prospective PNZ cells were removed before stage 24, no PNZ would be seen at stage 24 - there are no signals present at stage 24 which can induce a PNZ in non-determined cells.

Moreover, he found that prospective PNZ cells are working to a 'death clock' - if prospective PNZ cells are excised from a donor into culture or grafted into somitic tissue they die on schedule when the donor reaches stages 24.

Even when the prospective PNZ is excised from the wing and replaced with prospective PNZ from a donor of a different age, the PNZ dies when the *donor* reaches stage 24, independent of the age of the host within whom the graft now lives.

The death sentence is passed at stage 17, but until stage 21 it can be reversed by excising the prospective PNZ and placing it in the dorsal mesenchyme where cell death would not normally occur - a stay of execution provided by the dorsal limb mesenchymal signals. After stage 21 the cells cannot be rescued - grafted tissue dies *in situ* in dorsal mesenchyme at stage 24.

The rescuing factor from the dorsal mesenchyme is probably a diffusible protein, because in culture, prospective PNZ cells can be kept alive beyond donor stage 24, even if separated from the dorsal mesenchyme by a Millipore membrane (providing the pore size was greater than $0.05\mu\text{m}$) (Fallon and Saunders, 1968).

The cells are aware that they should be dead, and if removed from the proximity of the dorsal mesenchyme, die immediately. However, if they are cultured for more than 7 days before removal of the dorsal mesenchyme, the PNZ cells can chondrify and now no longer die when the mesenchyme is eventually removed.

Other workers (reviewed in Earnshaw, 1995) have recognised that apoptosis is divided into a 'sentenced' phase (when cells can be reprieved) and a 'condemned' phase, when they cannot. The nature of the gene expression differences between sentenced and condemned cells is not known.

Functions of cell death:

In non-experimental conditions, rescuing signals, e.g. from dorsal mesenchyme, would only occur if the limb was deformed or physically damaged. Thus the necrotic zones may have retained the ability to respond to rescuing signals as an aspect of developmental plasticity - helping to rescue an embryo which is going wrong.

Cell death *may* be important in eliminating unwanted tissue from the limb, such as that between the digits. Reduction in cell death between the toes of ducks correlates with the resultant webbing (Saunders and Fallon, 1966), and the lack of the ANZ in the moles, *Talpa*, correlates with the formation of an extra falciform digit. The anterior and posterior necrotic zones may act as end stops for the AER, since mice have a broader AER than chickens, correlating with smaller necrotic zones, and *talpid* mutant chickens, lacking ANZ and PNZ, also have an extended AER. *talpid* chickens also lack zones of interdigital cell death, leading to soft tissue syndactyly (Ede and Agerbak, 1968; Hinchliffe and Thorogood, 1974).

However, amphibians, although having well separated digits, appear to lack interdigital necrotic zones, and Saunders (1962) found that rescue of the PNZ did not result in ectopic digits. Cell death may not be indispensable in tissue modelling.

There are still questions to be answered about whether cell death is a consequence of cells receiving contradictory developmental instructions - a kind of default developmental decision in a confused cell. There is a need to investigate whether there are specific extrinsic or intrinsic signals which say 'die', or whether apoptosis is caused by intrinsic factors activated when incompatible extrinsic signals are received. If the latter is true, we want to know why some cells are susceptible to attempts to read two signals at once, when other differentiating cells can either ignore confusing signals or redifferentiate in response to them.

Bone and Cartilage Differentiation:

The tetrapod limb is derived from the sarcopterygian lobed fin, which employs an endochondrial skeleton. Thus the endochondrial skeleton supports the tetrapod limb, and there is now no contribution from the dermal skeleton which formed the fin rays.

Proximal limb bones start to form before the distal elements, which are developmentally younger and may not even be determined before the proximal elements start to differentiate (Summerbell *et al.*, 1973). The first morphological sign of skeletal differentiation is condensation of the mesenchyme - cells move

together, become 60% more closely packed (Ede and Flint, 1975a,b) and adhere tightly (Rosen and Thies, 1992). The factors determining placement of chondrifications are, as yet, unknown. Condensation occurs centrally in the limb; the epithelium can inhibit chondrification in the underlying mesenchyme (Solursh *et al.*, 1981, 1984). Any one chondrification centre may be able to inhibit chondrification in the mesenchyme immediately adjacent to it - possibly by means of a diffusible inhibitor. Thus the pattern of chondrification centres may depend on the *amount* of mesenchyme available at the *time* the cells decide to condense and chondrify (reviewed in Shubin and Alberch, 1986). There are a number of chicken and mice mutants where an extension of the AER leads to a broadening of the undifferentiated digit plate and hence to polydactyly (Coelho *et al.*, 1993c). Conversely, mutations which decrease the mass of undifferentiated mesenchymal tissue commonly lead to oligodactyly.

The exact shapes of the bones which result from the condensation process probably depend as much on local interactions which occur as the cartilage precursors condense as on any specified 3D co-ordinate prepattern. Ontogenetic development of the pattern of condensations characteristically (in tetrapods and sarcopterygians) occurs in three ways (Shubin and Alberch, 1986). (i) Some condensations arise *de novo*. (ii) As cells are recruited to the distal tip of a condensation during growth of the limb, single condensations may bifurcate distally into a Y-shaped element (as predicted by Turing's diffusion reaction model for pattern formation by morphogens). (iii) Alternatively, a single element may give rise to a single distal condensation (a segmentation event).

Embryonically connected condensations are separated by subsequent tissue cavitations resulting in joints. The formation of the knee joint in culture was treated in a series of experiments by Fell (1931). Cell death may aid the process of creating an empty zone for a joint; connective tissue then differentiates to form the joint capsule (Hinchliffe and Johnson, 1980). Prospective knee tissue from a stage 20 chick has the capacity to form a joint in culture, so there may be some joint-determined cells in undifferentiated limb mesenchyme.

The genetic basis of the timing of chondrification will be treated later.

Condensation is accompanied by increased cell adhesion as cells secrete new proteoglycans (the first of which is fibronectin (George *et al.*, 1993)) and chondroitin sulphate into the extracellular matrix. Precartilagenous cells need a signal from the surrounding mesenchyme (Solursh *et al.*, 1982)(at present unknown, but see later!) in order to start secreting the collagens and other materials which constitute cartilage. The proteoglycans link the chondroitin sulphate chains to the protein backbone. Chondrocytic cells express a battery of genes which distinguish them from the non-chondrocytic cells (Erlebacher *et al.*, 1995). Some proteins, such as type I collagen, are secreted only transiently during cartilage formation (Dessau *et al.*, 1980), whereas others, such as type II collagen, may not be secreted until later during the

process but are maintained in the mature cartilage (Linsenmeyer and Hendrix, 1992). In secreting these impermeable compounds, the chondrocytes sign their own death sentence, for they cut themselves off from the vascular system and hence their oxygen supply. Cells toward the centre of the bone cartilage model hypertrophy - they swell. The matrix between them becomes calcified and the cells die. Hypertrophic cartilage cells induce perichondrial cells to become osteoblasts, hence the cartilage becomes surrounded by a bony collar (Rosen and Thies, 1992). The internal hypertrophic cartilage is also replaced by bone and the honeycomb of calcified matrix is infiltrated by blood vessels, carrying phagocytes which clear away the dead cartilage cells (Rosen and Thies, 1992). Osteoblasts enter with the blood and form bone.

Osteoclasts also infiltrate the limb via the blood; they can resorb and reshape bone during life, and there is a delicate balance between the action of osteoblasts and osteoclasts, which can go wrong e.g. in osteoporosis. Ossification starts and is maintained at invariant, well defined, 'ossification centres' in each bone (Dolle *et al.*, 1993b). Short bones have one centre, the long bones characteristically have two or more, located in the centre and at either end just in from the epiphyses. It is these ossification centres which remain active throughout limb development into adulthood. Growth of cartilage and the ensuing ossification continues until, in adulthood, the ossification centre overtakes the epiphysis (in Hinchliffe and Johnson, 1980).

Creation of the pattern of chondrogenesis in the limb has led to conception of many morphogenetic theories, some of which will be considered later. Bone formation is a complex process and there are many unanswered questions.

Cartilage and bone are vertebrate-specific tissues and must have evolved *de novo* in the vertebrate line. What is the genetic basis behind bone formation? What does this tell us about how the tissue evolved - does it need vertebrate-specific genes? What is the genetic basis for bone patterning? Could the ancestral patterning genes control fully the pattern of chondrogenesis and osteogenesis? If so, what does this tell us about the evolution of new tissue types and new developmental pathways?

Muscle Differentiation:

The internal skeleton of the limb is mostly derived from the lateral plate mesoderm of the body wall. The clavicle is dermally derived, as in fish, and there is some contribution to the scapula from the sclerotome. In contrast, the limb muscles are entirely derived from precursor cells in the somites (Christ *et al.*, 1977).

Somites derive from the mesoderm which, after gastrulation, lies adjacent to the neural tube in the trunk and tail (Figure 1.7, A). In a rostro-caudal sequence, this presomitic mesoderm condenses into a defined number of epithelial balls - the somites - in pairs either side of the neural tube (Lasser and Munsterberg, 1994) (Figure 1.7, B). The medio-ventral quarter of these balls soon loses its epithelial morphology and migrates away in clumps. These cells, the sclerotome, under the control of genes induced by a signal from the notochord (Rong *et al.*, 1992; Goulding *et al.*, 1994) will form the axial skeleton - the ribs and the vertebrae. The remaining epithelial cells of the somite are the dermamyotome (Figure 1.7C). Cells at the dorsal lip of this sheet migrate over and down the inner surface of the dermamyotome; they are highly mitotic and they and their progeny form the myotome, becoming the axial muscles (Emerson Jr. 1993; Pownall and Emerson, 1992). The rest of the somite, the dermatome, will mostly contribute to the dermis, except for the cells at its ventrolateral edge. These proliferate and migrate away from the somite (either individually or, in reptiles, as a sheet) (Jacob *et al.*, 1978) and will form the musculature of the limbs and the body wall (Christ *et al.*, 1977, 1983; Ordahl and Le Douarin, 1992; Wachtler and Christ, 1994) (Figure 1.7D). Although most studies have been performed on chickens, Ede and El-Gedi (1986) showed that mice are the same as chickens in this respect. Contact between the medial cells of the presumptive limb somatopleure and the ventrolateral cells of the somite is necessary to initiate somite cell migration. (Hayashi and Ozawa, 1995)

In elasmobranch fishes, the contribution from the somites imposes segmentation on the fin - dermal rays condense in the space between the immigrating muscle sheets. This is not so in modern tetrapods, although there is a fixed number of somites which contribute to the limb (Hinchliffe and Johnson, 1980; Beresford, 1983).

The myogenic cells migrate into the limb bud, where they can be detected from very early stages. They are determined to become muscle (Tajbakhsh and Buckingham, 1994); they express *Pax3* prior to and during migration (Bober *et al.*, 1994; Williams and Ordahl, 1994), although they do not express any of the characteristic muscle specific genes, *MyoD*, *Myogenin* or *Myf5* until they are *in situ* in the limb (Ott *et al.*, 1991; Tajbakhsh and Buckingham, 1994). They aggregate, proliferate and form two groups of pre-muscle cells either side of the condensing long bones - the dorsal and ventral pre-muscle masses. They

continue to migrate distally, never quite penetrating the Progress Zone (Newman *et al.*, 1981; Rutz *et al.*, 1982).

By a series of bifurcations, the muscle masses give rise to all the muscles of the limb. A chick wing has 50 muscles and 30 associated tendons (in Hinchliffe and Johnson, 1980).

The dorsal and ventral pre-muscle masses probably represent the single abductor and adductor muscles which move the fish fin. Their bifurcation first produces, in mammals, a phylogenetically primitive muscle pattern which, by fusion of some pre-muscle blocks and death of others, then forms the final muscle pattern. Muscles are connected to the mesenchymally-derived tendons only when both have formed *in situ*.

There are two populations of early myocytes which express two different sets of myosin genes (Cusella De-Angelis *et al.*, 1994). One population differentiates first in a pattern directed by the mesenchyme and forms the primary myofibrils; they release mitogens and other factors (Cusella De-Angelis *et al.*, 1994) which cause the secondary myocytes to proliferate and then differentiate around the prepatter formed by the primary fibres. It is the secondary fibres which will produce 90% of the limb muscle

Although the myocytes are already determined to form muscle when they migrate from the somite, the exact muscle to which they will contribute has not yet been determined. Grafting them from a donor limb to a new position in a host limb causes them to differentiate into muscles (and nothing else) appropriate to their new position in the host.

Signals from the surrounding mesenchyme impose positional specification on the myocytes - grafting an ectopic ZPA anteriorly into the limb bud causes mirror-image duplications of muscles derived from host tissue, as well as skeletal elements. The muscle-specific patterns of slow and fast protein synthesis are also imposed by the surrounding mesenchymal connective tissue (Robson *et al.*, 1994).

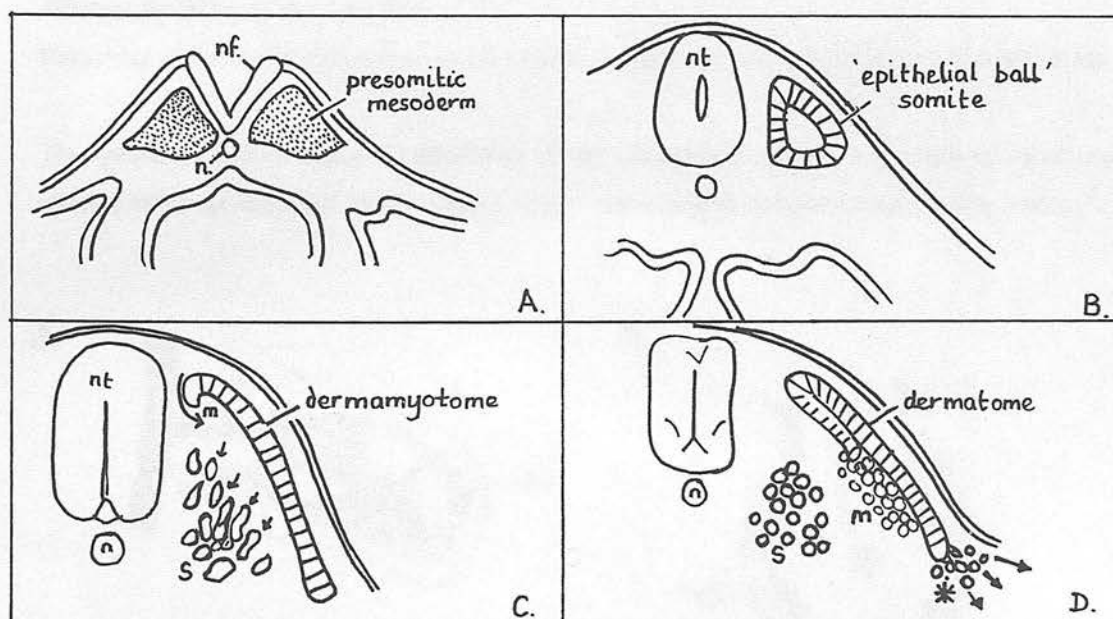


Figure 1.7: Development of somites, illustrated with transverse sections through the embryo. Arrows indicate cell movement. See text for details. Abbreviations: nf - neural fold; n - notochord; nt - neural tube; m - myotome; s - sclerotome. The asterisk (*) in the bottom right hand corner of D indicates prospective limb (or body wall) muscle cells budding from the ventrolateral dermamyotome.

Innervation:

In mice, innervation starts at around E11, when the spinal nerves of C4 to C8 and T1 migrate from the lateral motor column (Tsuchida *et al.*, 1994) into the fore-limb *via* the branchial plexus. Axons migrate *via* the lumbar plexus from L1-L6 to populate the hind-limbs (Martin, 1989). Side branches to the muscles and the skin form in a general proximo-distal progression, with ramification such that by E14.5, dense innervation reaches the fingers and toes. The nerves are guided to their targets by signals, possibly FGF-related proteins, which are released from the developing muscles (Gospodarowitz, 1987). Innervation occurs early - naked axons may migrate into position before the muscles they supply are fully differentiated. Neural crest-derived Schwann cells migrate in along the naked fibres (Grim and Christ, 1993). Nervous innervation leads to muscular twitching and contraction during development. This is necessary for the continued development of the muscles, without which they degenerate and apoptose. Embryonic movement and the resulting physical forces are necessary for formation of some secondary cartilages and sesamoid bones (those which occur in tendons under stress) (Hinchliffe, 1994; Erlebacher, 1995).

The developing limb contains many more nerves than it actually needs - a large number of nerve fibres apoptose, so that only the connections necessary for coordinate muscular control are retained.

As with muscles, the basic patterning of the limb can occur in the absence of nerves.

The vasculature:

Blood vessels, in common with muscles, neurons, Schwann cells, melanocytes (Grim and Christ, 1993), osteoclasts and chondroclasts, enter the limb secondarily. The endothelial cells of the capillaries form a rather uniform plexus within the early bud. The uniformity of this plexus is lost as gaps appear and the circulation of the blood is taken over by larger, fewer, veins and arteries, which branch to supply the different structures of the limb (Figure 1.8).

Branching of the blood vessels is as much a physical process of fluid dynamics as a biological one.

The lymphatic system arises independently of the vasculature; spaces in the mesenchyme acquire an endothelial lining and form vessels. These vessels make secondary connections with the veins.

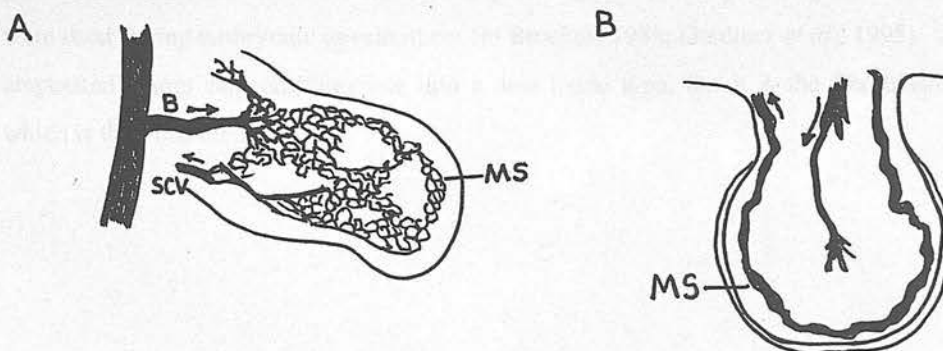


Figure 1.8:

A. Stage 26 chicken wing showing the capillary plexus reorganising into arteries and veins. The Brachial artery and subclavian vein are labelled. The marginal sinus (MS) is more pronounced in the mouse limb (B).

Skin Differentiation:

(from Davidson, 1978; Fuchs and Byrne, 1994)

Initially, the limb epidermis is simple cuboidal epithelium, derived from the embryonic ectoderm. Both during and after stratification there is rapid mitosis in all layers of the epidermis. The epidermis thickens to form placodes, with dermal condensations underlying them. There is a series of mesenchymal-epithelial interactions accompanied by changes in mitotic activity which results in epidermal cells differentiating and moving downwards into the follicles. They form a sheath which will produce the hair or feathers. As cells in and around the follicles die, they are replaced from a population of stem cells which may reside in the dermal papilla, the matrix or even amongst the melanocytes.

The choice of hair/scale/feather formation may reside primarily in the epidermis - feather formation can be induced in chicken corneal epithelium if cultured in contact with mouse flank dermis (Coulombre and Coulombre, 1971).

During development, the epidermal cells form a stratified epithelium and secrete increasing amounts of keratin, causing the cells to become flattened, hardened, and to die, when they are eventually shed. The cycle of shedding and replacement (by mitotic activity only in the basal layer of the epidermal cells) continues throughout life.

Limb regeneration:

The process of pattern formation and differentiation during limb development need not be restricted to the embryo. Certain urodeles have the astonishing property of being able to regenerate a new limb (or tail) from the stump of an amputation in the adult (larval anurans may have the same ability) (Brockes, 1989 review; Muneoka and Sassoon, 1992). After amputation, the wound heals; epidermal tissue grows round from the stump and piles up forming an Apical Ectodermal Cap, AEC. Damaged or traumatised cartilage, muscle and connective tissue release mononucleate cells into the covered wound. There is a period of phagocytosis as cell debris is cleared away, and the surviving tissue then dedifferentiates - cells from different tissue origins become morphologically indistinguishable from each other; they become a mesenchymatous, mitotically active blastema (essentially a new Progress Zone), beneath the AEC (Muneoka and Sassoon, 1992; Gardiner *et al.*, 1986). As the blastema proliferates, the whole process of pattern formation and tissue differentiation occurs again - possibly employing the same processes which were used during embryonic development (in Brockes, 1989; Gardiner *et al.*, 1995). All cell types in the amputated stump can redifferentiate into a new tissue type, but it is the fibroblastic connective tissue which is the most metaplastic.

Neurons regenerate by outgrowth of the cut axons. They release a chemical which is absolutely required to prevent necrosis in the stump and to induce blastema formation (Muneoka *et al.*, 1992).

It is the blastemal mesenchymal tissue which appears to direct pattern formation, and will direct the development of the appropriate structure (i.e. hind-limb or fore-limb) even when transported to a different part of the body.

Reptiles and mammals have only very limited powers on limb regeneration. The ability of adult urodeles to regenerate limbs may be associated with the continued expression of some homeobox-containing genes in the limbs of the adults (Beauchemin and Savard, 1992; Simon and Tabin, 1993; Tabin 1989).

The role of homeobox-containing genes in development suggests they would have a role in developmental plasticity and redifferentiation. We can ask what is the true genetic basis behind the property of 'plasticity' - both in the adult and in the embryo. If it is true that maintenance of homeobox gene activity is necessary for the maintenance of plasticity, then the question is what is special about maintenance - why can cells not reinitiate homeobox genes in response to damage? What has happened at the molecular level to terminally differentiated cells?

From Needles to Gilsons - molecular analysis of limb development:

Limb development is under genetic control, normally with little or no environmental influence - the limb is an adaptation to, not a consequence of, the environment. An understanding of the control of limb development requires an understanding of the molecules which are employed during pattern formation in the limb.

Retinoids in limb development:

A large amount of work has investigated the effects of retinoids on limb development (reviewed in Brookes, 1989; Tabin, 1991). Attention centred for a long time on the fact that an agarose bead, soaked in all-trans retinoic acid or 5,6-didehydroxyretinoic acid and implanted into the anterior margin of the limb bud can, in cooperation with the AER, mimic the effect of grafting an ectopic ZPA into the anterior margin - a mirror-image digit duplication (Tickle *et al.*, 1982; Thaller *et al.*, 1993).

Retinoic acid was a candidate morphogen - a molecule which could be released from the ZPA forming a posterior-to-anterior concentration gradient which could be 'read' by limb mesenchyme cells in order to determine their position along this axis and to differentiate appropriately.

What made this hypothesis plausible was that in chick limbs, a posterior-anterior gradient of both RA and 5,6-ddRA was reported (Thaller and Eichele, 1987; Tickle and Brickel, 1991). These gradients were small, (about three times higher posteriorly than anteriorly) but accentuated by counter gradients of cellular RA binding proteins (CRABPs) anterior-posterior (Smith *et al.*, 1989). These cytoplasmic proteins bind RA and sequester it from the nucleus. RA effects cells when it binds to RARs and RXRs - DNA-binding transcription factors of the class which, like the steroid receptors, is only active when it dimerises and binds a ligand - in this case, retinoids.

There are two families of retinoic acid receptors - RARs which bind all-trans RA and 9-cis RA (RAR α 1, α 2, β 1-4, γ 1, γ 2); RXRs (RXR α , β , γ) which bind only 9-cis RA (Mendelsohn *et al.*, 1992). The different family members and isoforms of the genes show different but overlapping expression patterns in the embryo. They probably form heterodimers with each other to exert their biological effect (RXRs probably also form heterodimers with other steroid receptor types) and there is a degree of functional redundancy between them (Kastner *et al.*, 1994; Thaller *et al.*, 1993). RARs α , β and γ are expressed in the developing limb in patterns which suggest they have a morphogenetic role (Mendelsohn *et al.*, 1992; Tabin, 1991). In particular, expression of RAR β is associated with interdigital cell death and cell death between the long bones (the opaque patch), as are the CRABPs (Gustafson *et al.*, 1993). In contrast,

RAR γ , after being uniformly expressed in the early limb bud, becomes restricted to the cartilage cells of every bone model. RAR α is expressed in the Progress Zone of the early limb bud.

The case for RA being a morphogen now looks quite shaky, and indeed there always was evidence against it. For example, although grafting an RA bead into the limb causes upregulation of RAR β in neighbouring cells, implantation of an ectopic ZPA does not, suggesting that RA secretion is not a function of the ZPA (Tabin, 1991). Secondly, mirror-image duplications can be induced in amphibian eggs incubated in low concentrations of RA, suggesting that a concentration gradient is not necessary for the duplication effect (Tabin, 1991). Recent work has shown that there are large concentration differences in different retinoids between mouse and chicken, and that there is no correlation between the concentration of the retinoids and their binding proteins (Scott Jnr. *et al.*, 1994). The evidence for a genuine concentration gradient of the forms of RA across the limb is also shaky - RA probably localises to the periphery of the limb, not in the chondrogenic core (Scott Jnr. *et al.*, 1994). Moreover, the absolute concentration of RA in the limb bud is at a minimum when polarising activity is at a maximum (Tabin, 1991). The real nail in RA's coffin had come earlier however. Wanek *et al.* (1991) showed that RA can induce polarising activity in anterior limb tissue - an RA bead was implanted into the anterior of a stage 21 chick limb bud, then after 24 hours tissue from the distal edge adjacent to the bead was extracted from the limb and implanted into the anterior of another stage 21 chick limb. This limb developed mirror-image duplications. No RA had been carried over into the duplicated wing; the mesenchyme adjacent to the AER in the limb into which the bead was implanted had been giving polarising activity.

This experiment, together with the effect in the regenerating newt blastema (it causes proximalisation and posteriorisation of the tissues (Brockes, 1989)) and the observations of the teratogenising effects of RA in other parts of the body, suggested the real role of RA - it is causing a posterior respecification of mesenchymal tissue in the limb and hence creating a new ZPA.

This implies that RA should induce expression of genes associated with the posterior margin of the limb bud which it has been shown to be able to do (Olivier *et al.*, 1991; Ohsugi and Ide, 1993; Koyama *et al.*, 1993).

From spanners to cogs - *in vivo* limb signals:

One such gene is *Sonic hedgehog*, *Shh*; RA, in conjunction with a signal from the AER, can induce the expression of *Shh* in limb tissue. This evolutionarily conserved gene is normally expressed in the posterior of the limb bud in a domain which matches that of the empirically defined ZPA (Chang *et al.*,

1994; Riddle *et al.*, 1993). It is also expressed in the primitive node, notochord and floor plate of the neural tube - areas which themselves have polarising activity and which can cause mirror-image duplications if grafted into the anterior margin of the chick limb bud (Wagner *et al.*, 1990; Hornbruch and Wolpert, 1986). Its *Drosophila* homologue, *hedgehog*, has been shown to be a signalling molecule which, in different situations, can either act as a short range cell-cell signaller, or as a long range morphogen (Peifer, 1994; Heemskerk and DiNardo, 1994).

All the properties of the ZPA (Koyama *et al.*, 1993):

- (i) found in the posterior margin of the limb;
- (ii) activity is conserved between species (Krauss, 1993);
- (iii) activity also found in notochord, primitive node and neural plate (Echelard *et al.*, 1993; Roelink *et al.*, 1994);
- (iv) diffusible signal;
- (v) induced by retinoic acid in conjunction with the AER (Helons *et al.*, 1994);

are also properties of *Shh*, making it a strong candidate for the postulated ZPA morphogen. Cell lines which do not have polarising activity in the limb gain this property upon induction of expression of *Shh*.

It has been mentioned that RA has its posteriorising effect in conjunction with a signal from the AER and that the AER is necessary for the maintenance of the ZPA. There are many genes expressed in the AER which could potentially act as signals e.g. *Wnt5a*, *Bmp2*, *Bmp4*, *Fgf2*, *Fgf4*.

One of these genes, *Fgf4* (fibroblast growth factor), which is expressed in the posterior AER, has been shown to have a biological AER-like effect. If the ectoderm is removed from a stage 21 chick wing bud *in ovo*, limb outgrowth and development normally stops; if, after AER removal, an *Fgf4*-soaked bead is placed at the distal tip of the mesenchyme (mimicking the AER), and an RA bead posteriorly, an almost normal limb was produced (Vogel and Tickle, 1993). Implanting only one of the beads leads to deformed limbs - a molecular reproduction of the experiments which showed that both an AER and a ZPA are necessary to maintain a Progress Zone and that the three zones are dependent on each other for their maintenance. Recent experiments have shown that *Fgf4* and RA are both necessary to induce *Shh* expression in experimental situations (although *Fgf4* on its own can maintain polarising activity), which then feeds back to maintain *Fgf* expression in the AER, which in turn maintains *Shh* expression (Niswander *et al.*, 1994; Vogel and Tickle, 1993; Helms *et al.*, 1994). Hence we have a molecular framework which could, in part, explain the interdependence of the three limb zones on each other.

In terms of limb development, we would want to ask whether the *Fgf4/Shh* interaction which maintains polarising activity is the most upstream pattern-forming mechanism in the limb. There must be genes upstream which create the initial limb asymmetry which allows the *Fgf4/Shh* system to be set up. Another way to look at this question is to ask what aspects of the retinoic acid response pathway prime cells to be able to respond to Fgf4 by expressing *Shh*?

The *Hox* genes may provide the initial asymmetry. The normal expression domain of *Hoxb8* along the anterior-posterior axis encompasses the posterior-proximal corner of the emerging limb bud. A transgenic mouse which also shows ectopic expression in the anterior of the fore-limb bud develops ectopic digits arranged symmetrically across the anterior-posterior axis. *Hoxb8* may be involved in setting up the polarising activity of the ZPA (Charite *et al.*, 1994, but see also Chan *et al.*, 1995). This would also provide a link between the primary a/p axis and the secondary axis of the limb.

Hox genes in limb development:

The *Hox* genes have a role in limb development. As influencers of the development of the rostro-caudal axis it is likely that they are determinants of the areas in the body wall from which the limbs will grow. In a *Hoxb5* knockout mouse, the shoulder girdle shifts forward with respect to the axial skeleton by one vertebra, suggesting that the position of the shoulder girdle is specified by the *Hox5/6* expression boundaries (Rancourt *et al.* 1995). *Hox* genes almost certainly also have a role in the arm/leg decision.

All four *Hox* clusters are represented in the limb. *Hoxd9*, *d10*, *d11*, *d12* and *d13* are expressed in the mesoderm of all limbs (Duboule, 1994; Izpisua-Belmonte and Duboule, 1992), as are *Hoxa9*, *a10*, *a11*, *a12* and *a13* (Haack and Gruss, 1993). *Hoxc9*, *c10*, *c11* and *c12* are expressed in the mesoderm of the hind-limbs (Peterson *et al.*, 1994). The data for the *Hoxb* complex is incomplete, but *Hoxb8* is expressed in the fore-limb (Charite *et al.*, 1994).

The *Hoxc6* homologue of *Danio* is expressed in an anterior-proximal gradient in the mesoderm of the fin bud in an expression domain equivalent to that seen in frogs and mice (Molven 1990; Izpisua-Belmonte and Duboule, 1992). This, together with the expression of *Shh* in the posterior fin bud mesenchyme (Kraus *et al.*, 1993) is good molecular evidence that fins are homologous to limbs and that the patterning mechanisms which limbs use were to some extent already set up in fish.

The genes expressed in the limbs are those at the 5' end of each cluster which are expressed most caudally along the primary axis. Whereas the hind-limb expresses genes in areas which are consistent with their expression patterns along the primary axis, the fore-limb does not. It has been suggested that some form of homeotic transformation occurred early in the vertebrate lineage, creating a fore-limb which is essentially similar to a hind-limb because it is expressing the same *Hox* genes (Ahlberg, 1992; Tabin, 1993). Only sarcopterygians and their descendents show fore- and hind-limbs which consistently share a common plan. If the homeotic transformation occurred at all, it must have been early in the sarcopterygian line, after the split with the actinopterygians (Coates, 1994). This argument of course ignores the fact that sarcopterygian tail fins are well patterned and may themselves be expressing *Hox* genes during development.

In common with their expression along the primary axis, the 3' genes are induced before their 5' neighbours. Hence, as the limb grows out, *Hoxd9* is turned on first, then *Hoxd10* in the posterior-distal corner of the limb, then *d11*, then *d12* then *d13* forming nested domains of expression which are initially arranged along the anterior-posterior axis of the limb (Dolle *et al.*, 1989; reviewed in Duboule, 1992). The interaction between the AER and the ZPA mediates the sequential turn-on of these genes (Koyama, 1993; Izpisua-Belmonte and Duboule, 1992).

Tabin (1992), on the basis of some cartoon data and a dodgy fate map proposed that the different combinations of *Hox* gene expression turned different areas of the handplate into different digits, i.e. *Hoxd9* only = digit 1; *Hoxd9* and *d10* = digit2; *Hoxd9*, *d10* and *d11* = digit 3 etc. The fact that early tetrapods could have more than 5 digits was explained by saying that they had only five *types* of digit.

Evidence was provided for the model by ectopically expressing of *Hoxd11* throughout the chicken limb, which, as predicted, caused an apparent homeotic transformation of digit 2 into digit3 (Morgan *et al.*, 1992).

When it was shown that the *Hoxa* complex was expressed in nested domains arranged along the proximo-distal axis of the limb (with 5' genes being expressed latest and most distally) (Yokouchi *et al.*, 1991; Haack and Gruss, 1993) it was thought possible that the two sets of domains could form a 2-D patterning mechanism for the bones of the limb - each condensation could have a different *Hox* code which determined which bone it developed into.

Further data has not supported this model. Firstly, as the limb bud grows out, the *Hoxd* genes become arranged in a proximo-distal sequence like the *Hoxa* genes, and become quite complex expression patterns which cannot be related to anterior-posterior specification (Davis and Cappechi, 1994) (Figure 1.9). This may be a consequence of the posterior bias in the fate map of the limb which was referred to when discussing the evolution of the limb. Cells in the posterior of the limb divide and grow more quickly than

those in the anterior and populate the anterior margin like toothpaste out of a tube - they may push the domains of the *Hoxd* complex round with them (Duboule, 1994).

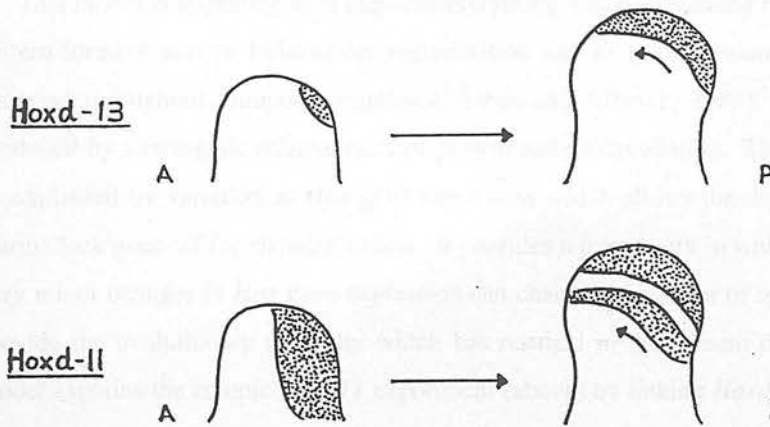


Figure 1.9: Changes in *Hoxd* expression patterns during limb development. The change from early anterior-posterior restriction in the handplate to later proximal-distal does not occur in *Danio* (Sordino *et al.*, 1995) which may provide molecular evidence for the neomorphy of digits. A - anterior; P - posterior.

Secondly, the first *Hox* knockout to affect the limb was that of *Hoxd13* (Dolle *et al.*, 1993). The model proposed above suggests that this would transform digit 5 into digit 4, but this did not happen. Digits 2 and 4 had phalanges missing, other digits had foreshortened phalanges and some of the carpals were deformed. *Hoxa11* and *Hoxd11* knockouts followed (Davis and Cappechi, 1994; Small and Potter, 1993), which had similar effects on malformations of individual bones which would not have been predicted by the above model. The *Hoxa11/Hoxd11* double knockout has no radius or ulna (Davis *et al.*, 1995). In the absence of further data, it looks like the mutations in the 3' genes have effects more proximally in the limb than the 5' gene. These mutations manifest as delays in condensation and chondrification of individual skeletal elements, followed by delays in ossification of the cartilage models (Davis and Cappechi, 1994; Dolle *et al.*, 1993; reviewed in Graham *et al.*, 1994).

This, together with some effects of gene knockouts on the axial skeleton, is consistent with the timing of condensation of the cartilagenous precursors being altered with regard to the size of the limb bud, and hence the amount of mesenchyme available to condense. In the knockouts the mesenchyme may be condensing while the digit plate is too small, leading to reductions and malformations in the bones. The implication is that the *Hox* code in the limb is related to the clock which times the differentiation process, and may be related to the Progress Zone clock which specifies elements along the proximo-distal axis.

Hox genes may be the mechanistic link between growth and patterning, ensuring that the two processes remain in synchrony (Duboule, 1994).

This model is appealing as it explains everything whilst explaining nothing. Assuming that the basic pattern-forming acts of bifurcation, segmentation and *de novo* condensation of skeletal elements have occurred throughout tetrapod evolution (Shubin and Alberch, 1986), then any observed limb can be produced by altering the relative rates of growth and differentiation. The seven digits of *Ichthyostega* can be explained by variation in *Hox* gene expression which allows the digit plate to get bigger before the alarm clock goes off for chondrification. It provides a framework in which it is easy to conceptualise that very minor changes in *Hox* gene expression can change the pattern of formation of the bones, and hence provide the evolutionary plasticity which has resulted in the present diversity of vertebrate legs. The model explains the ectopic *Hoxd11* experiment (above) by linking *Hoxd11*'s expanded ectopic domain to an increase in proliferation in the anterior mesenchyme, leading to more tissue being available to condense, resulting in extra phalanges for digit two (Morgan and Tabin, 1994).

Until there is more data from *Hox* gene knockouts, and until there is some experimental evidence that limb cells expressing different *Hox* genes differentiate on a different timescale, it would be unwise to say too much about this model. It is at present little more than a hypothesis.

Even if the model were shown to be correct, the expression of the *Hox* genes would still not fully explain pattern formation and morphogenesis during limb development. Indeed, the *Hox* genes seem to ask more questions than they answer.

Firstly, although the simple homeotic 'five genes for five fingers' model can be discounted on the basis of the knockouts, the expression of ectopic *Hoxd11* throughout the chicken limb does result in a homeotic mutation. If the extra phalange is purely a passive result of a mis-timing of the differentiation process, resulting in extra amounts of mesenchyme condensing, then why are other apparent homeotic mutations not similarly explicable? What is the difference between a C7-T1 vertebral transformation and a deformation of the cervical vertebra resulting from a change in the relationship between proliferation rate and condensation of the spinal processes? I would argue that there is none, and that evidence from the limb reinforces the explicable nature of homeosis in terms of defineable cell activities.

Secondly, there must be other genes involved in the process of cell-fate determination within the limb. If the *Hox* genes control only the rate of proliferation and timing of differentiation, and the ZPA imposes some positional information we are still left with the question, why do different cells, which may be next to each other in the bud, undergo different eventual fates (e.g. perichondrium/ligament/fibroblast)?

Different cells must be responding to the same extrinsic signals in different ways - are there other genes acting intrinsically which specify eventual cell fate rigidly? Since cell-cell interactions have roles in fate determination of the cells which interact, we also want to know what the genetic nature of these interactions is, and can we hence explain why the limb is a self-contained developmental system?

There must be many genes involved in limb development between the levels of *Hox* gene expression and cell fate determination. Do any of these genes contain homeoboxes, and can we build up a more complete picture of the genetic interactions which build the limb?

Homology between gene expression in vertebrate and invertebrate limbs:

Evidence presented earlier suggested that there appears to be some conservation of expression domains and functions between the developmental genes of *Drosophila* and their vertebrate homologues.

The possibility is therefore raised that genes which are expressed during the development of the Arthropod limbs may also be expressed and/or have similar functions in vertebrates. I am neither knowledgeable nor ambitious enough to attempt to describe all the genes expressed in the *Drosophila* wing or limb imaginal discs. This much I do know, however:

Cells in the posterior compartment of the wing or limb imaginal disc express the segment polarity gene, *engrailed*. Expression of this gene marks cells as 'posterior' and more-or less prevents them or their descendants from straying into the anterior compartment of the limb (Hidalgo, 1994). *engrailed* upregulates the transcription of *hedgehog* in these cells; hedgehog is released from the cells and diffuses a short distance through the extracellular matrix, such that cells in the anterior compartment of the disc on the border with the posterior compartment are exposed to hedgehog protein (Basler and Struhl, 1994; reviewed in Campbell and Tomlinson, 1995). Cells at the dorsal a/p border are competent to express *decapentaplegic*, *dpp*, and do so in the presence of hedgehog; the hh receptor is probably the product of the *patched* gene (DiNardo *et al.*, 1988). Similarly, cells at the ventral a/p border express *wingless* upon receiving the hh signal (Panganiban *et al.*, 1994). Cells at the meeting point of the *wingless* and *decapentaplegic* stripes of expression initiate a set of distal genes such as *aristaless* (Campbell *et al.*, 1993) and *distalless* (Diaz-Benjumea *et al.*, 1994; Panganiban *et al.*, 1994) and it is these cells which will

grow out to form the length of the limb - the bullseye of the polar coordinate clock (French *et al.*, 1976) or the crossing point of the boundaries (Meinhardt, 1983) (Evidence from both *Drosophila* and mice has suggested that boundary models of proximo-distal axis formation may be accurate, and that the crossing point of two or more boundaries of gene expression may be the trigger for distal outgrowth.)

I have described that a vertebrate homologue of *hedgehog*, *Shh*, is expressed in the developing vertebrate limb and that it may be acting as a signalling molecule. Homologues of *wingless*, *decapentaplegic*, *engrailed* and *distalless* are also expressed in vertebrate limbs.

***decapentaplegic* homologues:**

decapentaplegic is a member of a superfamily of TGF β related signalling molecules (reviewed in Kingsley, 1994). This superfamily has homologues and related molecules in apparently all species of animal, and many are found in vertebrates, e.g. the TGF β subfamily, activins, Vg1, *dorsalin*, *nodal*, 60A, the bone morphogenic proteins (BMPs). *dpp* is part of the DVR group of molecules which includes the BMPs2-8, Vg-1, *dorsalin* and 60A. In particular it is most closely related to the two vertebrate BMPs, *Bmp4* and *Bmp2*. Both these genes are expressed in the developing vertebrate limb, and their patterns of expression are known for mice and chickens (Francis *et al.*, 1994; Jones *et al.*, 1991).

Bmp2 is expressed during the pattern-forming early stage of the limb (Francis *et al.*, 1994). It is found throughout the AER and in a posterior domain in the mesenchyme which correlates with, though is slightly more extensive than, the ZPA and the domain of *Shh* expression (Johnson *et al.*, 1994). *Shh* has been shown to induce *Bmp2* and, as *Bmp2* is a diffusible signalling molecule, it remains possible that this is one ZPA signal (Laufer, 1994a), although expression of this gene on its own is not sufficient to confer polarising activity (Francis *et al.*, 1994).

In vertebrates, as in the insect limb, a *hedgehog* protein induces a *dpp* protein, apparently to impose positional specificity on the limb.

This is the best example I have, however, of this sort of conservation of gene activity and interactions across the chasm of evolutionary time. *Bmp4* is expressed in a pattern in the limb bud which does not suggest an association with *Shh*. It is expressed at the same time as *Bmp2*, also throughout the AER, but in the mesenchyme it is expressed in the non-chondrogenic marginal tissue, anterior, posterior, distally in the Progress Zone and in the areas of interdigital cell death. There will be a full discussion on *Bmp4*, and other Bmps, in Chapter Eight.

***wingless* and *engrailed* homologues:**

Of the other genes expressed during the patterning of the insect limb, *wingless* has homologues which are expressed in vertebrates (Siegfried and Perriman, 1994). *wingless* encodes a secreted glycosylated polypeptide, now known to act as a cell-cell signalling molecule (Van de Heuvel *et al.*, 1989; Vincent and Lawrence, 1994). *wingless*, in common with a number of developmentally-important genes, appears to have undergone a series of duplication events early in the vertebrate line (Holland, 1994; Ruddle *et al.*, 1994), with the result that there are at present 11 vertebrate homologues known - the *Wnt* family. They are expressed in many areas of the embryo; six of them are found in the limb (Parr *et al.*, 1993).

Wnt5a is the only one expressed in limb mesenchyme - it is found in the Progress Zone and is also expressed in the interdigital necrotic zones and the perichondrium of the phalanges (Gavin *et al.*, 1990). It is also expressed throughout the AER.

Wnt3, *Wnt4*, *Wnt6* and *Wnt7b* are expressed uniformly in limb ectoderm (Parr *et al.*, 1993).

Wnt7a is expressed in the dorsal ectoderm and has been shown to be a dorsalisating factor for the limb. Homozygous knockout mice show double-ventralised paws in which the dorsal half of the paw has pads, with skin, muscle, bone and tendon structure mirroring the ventral half (Parr and McMahon, 1995). *Wnt7a* also cooperates with *Fgf4* to induce *Shh* in the posterior of the limb - removing the dorsal ectoderm leads to downregulation of *Shh* which can be rescued by grafting a block of *Wnt7a*-expressing cells into the dorsal mesenchyme (Yang and Niswander, 1995). This is why *Wnt7a* knockout mice often have posterior limb structures missing and shows that the specification of prox/dist, a/p and d/v axes is interdependent.

This data provides molecular confirmation of the observation that surgically altering the dorso-ventral orientation of the ectoderm of the developing limb changes the dorso-ventral orientation of mesodermal structures subsequently specified

One of the vertebrate *engrailed* homologues, *En1*, is expressed in the ventral ectoderm (Davis *et al.*, 1991; Davidson *et al.*, 1988) - raising the possibility of cell-cell signalling via a *Wnt/En* pathway in the manner of *wg/en* in the *Drosophila* larval ectoderm. *En1* is presumed to have a role in normal limb development, since *En1* knockout mice show ectopic bones and skeletal abnormalities in the ventral regions of the paw (suggesting that the anti-chondrogenic properties of the ventral ectoderm have been lost). (Solursh, 1984; Wurst, 1994)

Abnormal ectopic expression of *Wnt1* in the limbs of transgenic mice produces a phenotype with limb deformities characterised by abnormal chondrifications - the *Wnt* genes can be a spanner in the works of limb development (Zakany and Duboule, 1992).

***distalless* and *evenskipped* homologues:**

A vertebrate *distalless* homologue, *Dll1*, (one of eight genes) is expressed in the posterior Progress Zone (Dolle *et al.*, 1992), eerily in the zone of cells where the *Bmp2* (*dpp*) and *Wnt5a* (*wg*) signals meet! *Evx1*, a vertebrate homologue of *evenskipped*, is also expressed here.

Recently, it has been demonstrated that the expression of *Evx2*, located 15kb 5' of the *Hoxd* cluster shows an expression pattern which would (on the basis of sites of expression, spatial and temporal colinearity) be predicted of a hypothetical *Hoxd14* gene - in the limb, its expression domain is nested within that of *Hoxd13* (Dolle, 1994). *Evx1*, located 45kb 5' of the *hoxa* cluster, shares much less expression similarity with the 5' *Hoxa* genes. The best explanation for this data is that *Evx2*, and, to a lesser extent, *Evx1* are falling under the influence of *Hoxd* regulatory elements.

Fibroblast Growth Factors:

After the BMPs and the Wnts, the other major family of cell-cell signalling molecules found in the limb is the fibroblast growth factors, FGFs - another multi-membered family of closely related molecules (Goldfarb, 1994). They were named because of Fgf2's (bFGF) ability to cause proliferation and transformation in 3T3 cells (Gospodarowitz *et al.*, 1987). There are at least 9 vertebrate FGFs, of which *Fgf2*, *Fgf4* and *Fgf8* are expressed in the limb (Suzuki *et al.*, 1992; Niswander and Martin, 1992; Crossley and Martin, 1995). *Fgf4* as stated previously, is expressed in the posterior AER and can substitute for the AER in experimental situations (Niswander and Martin, 1993) suggesting it has a genuine developmental role. It also has a positive effect on the growth of limb buds cultured without their ectoderm (Aono and Ide, 1988; Wang and Sassoon, 1995), in contrast to *Bmp2* which has a negative effect (Niswander and Martin, 1993). *Fgf2* is expressed throughout the AER and rather uniformly throughout the mesoderm of the early bud (Savage, 1993). It certainly has an active biological role as an AER signal (Fallon *et al.*, 1994), and is an inducer of proliferation in the mesenchyme (as are *Fgf1* and *Fgf5*) (Laufer, 1994) - *Fgf2* needs to be above a certain threshold concentration to maintain proliferation in the limb bud mesenchyme. *Fgf2* can also induce partial duplications when introduced to the anterior margin of a stage 21 chick wing bud. Only the anterior/distal structures of the bud can be duplicated, and the biological basis of this property is unknown, although it seems that a new Progress Zone is induced to form the duplicated structures (Riley *et al.*, 1993).

Fgf2 is also expressed in myogenic cells migrating into the limb bud, and some differentiating limb muscles (Joseph-Silverstein *et al.*, 1989).

The FGFs are implicated in having a role in limb bud formation. Cohn *et al.* (1995) showed that ectopic limbs could be induced by implanting beads soaked in FGF1, FGF2 or FGF4 into the flank mesenchyme of the limb field of stage 14 chicken embryos. Mima *et al.* (1995) observed the same effect by infecting the flank with a retroviral vector expressing *Fgf4*. Wings tended to grow out from the anterior flank and legs from the posterior, suggesting that the flank is already specified as 'arm' or 'leg' at this point. The limbs usually showed reversed polarity, suggesting that FGF stimulates proliferation and outgrowth, and also that it induces *Shh* in cells with polarising potential. Once the basic, polarised bud is set up, the limb is self-sufficient for further development, it can pattern itself and stimulate immigration of muscles, tendons and nerves.

Four FGF receptors have been identified, and at least 3 of them are expressed in the limb, in different, but partly overlapping, expression patterns which suggest they may be involved in limb morphogenesis. FGFR1 is found exclusively in non-chondrogenic limb mesenchyme, in direct contrast to FGFR2, which is exclusively chondrogenic. FGFR3 is found in some of the cartilage models and also weakly in the surrounding mesenchyme (Peters *et al.*, 1992; Noji *et al.*, 1993; Orr-Urtreger *et al.*, 1991). Defects in FGFRs are responsible for Apert, Crouzon and Pfeiffer syndromes (Wilkie *et al.*, 1995a,b; Rutland *et al.*, 1995; Mulvihill *et al.*, 1995) and are the best candidates for some of the mouse *luxoid*-type mutations.

Whither now?

Whither now? 30 years after the discovery of the PZ and the ZPA, we at least have a conceptual framework of how they function in terms of the activity of genes encoding the FGFs, BMPs, Hox proteins etc. Future progress in the genetics of limb patterning seems likely to come from a greater understanding of the roles of those genes which help to position the chondrification, muscles and connective tissue - the genes involved in the tissue-tissue interactions which appear to define the limb pattern. The expanding knowledge of the roles of molecules such as BMPs, FGFs and Wnts in other parts of the body may define the interactions in which they are involved in the limb - witness *Shh* and maybe *Bmp4*. Much of the development of the pentadactyl limb seems destined to be a black box for years to come, but there are some obvious candidate genes for immediate experimental investigation, not least of which are those of the *msh*-class.

THE MSH-CLASS HOMEBOX-CONTAINING GENES

Introduction:

A lot is known about homeobox genes and a lot is known about the limb, but there are a lot of unanswered questions. What mediates the response of cells to the instructions from the *Hox* genes about proliferation and differentiation? What is the molecular basis of the ability to respond to these intrinsic, and extrinsic, signals which push cells into canalised developmental pathways? Alternatively, what is the genetic basis of inhibition of differentiation and of the ability to redifferentiate (developmental plasticity)?

There is also scope for studying how the evolution of the homeobox-containing genes may have initiated the evolution of the vertebrate limb *per se*, and of the vertebrate-specific tissues within the limb (e.g. cartilage and bone).

What tissue-specific interactions occur which elaborate the patterning process during limb development; what homeobox genes are involved and are any of them responsible for the differentiation of specific tissues or for specific cellular processes; are they all one step removed from the final determinative cell fate decision? Do different classes of homeobox genes show similar control mechanisms, or are there differences which can be related to the evolution of the limb?

Studying the control and function of the vertebrate *msh*-class homeobox-containing genes provides the opportunity to start answering some of these questions. The *msh*-like genes are another class of homeobox-containing genes which underwent duplication events early in the vertebrate line. This potentially gave them special role in the evolution of vertebrate idiosyncracies such as the limb.

Discovery of the *msh*-class genes:

Muscle segment homeobox (originally *muscle-specific homeobox*), *msh*, is a *Drosophila* homeobox-containing gene, isolated on the basis of cross-hybridisation with *Ubx* (McGinnis *et al.*, 1984). It has a complex and dynamic expression pattern which includes the pharyngeal, somatic and visceral musculature, stomodeum and proctodeum, and subsets of neuroblasts in the CNS and PNS (Gehring, pers. comm.).

It is not closely related to the *tinman* gene, *msh-2*.

Homologues of *msh* have been found in the vertebrates. Hill *et al.* (1989) screened a λ gt10 cDNA library from 8.5d mouse embryos using the *Hoxa1* and *ftz* cDNAs as probes. He selected clones which hybridised with low intensity to *Hoxa1* and undetectably to *ftz*. Subcloning and sequencing the insert from such a clone revealed that it contained a cDNA for a homeobox-containing gene with 57%

homeobox sequence similarity to *Hoxa1*, 55% similarity to *engrailed* but 80% similarity to *msh* (90% similarity at the protein level). Genetic mapping placed the gene on chromosome 5, away from the other clusters and hence it was called *Hox-7.1*. Rescreening the library with *Hox-7.1* cDNA revealed a second unlinked gene with 85% sequence similarity which became *Hox-8.1* and a third unlinked gene (to become *Hox-9*). This is therefore a family of vertebrate homeobox-containing genes which are related to *Drosophila msh* (At the same time, Robert *et al.* (1989) isolated *Hox-7.1* by screening a mouse embryonic library with an *msh* probe). Since the term 'Hox' has subsequently been reserved for the *Antp*-like clusters, *Hoxa*, *b*, *c* and *d*, the genes have been renamed *Msx1*, *Msx2* and *Msx3* respectively. Homologues of these genes are found in humans (*MSX1*, *MSX2* at least (Hewitt *et al.*, 1991; Suzuki *et al.*, 1993)), chickens (*GHOX-7*, *GHOX-8*), quails (*QHOX-8*, *QHOX-7*), zebrafish (*msha*, *mshb*, *mshc*, *mshd* and *mshe* (Holland, 1991; Akimeto *et al.*, 1995)) as well as *Xenopus* (*Xmsh1* and *Xmsh2* (Su *et al.*, 1991)), *C.elegans*, Cephalochordates, Urochordates (reviewed in Holland, 1994), Bumble bees (Walldorf, 1991), echinoderms (Bell *et al.*, 1993) and the Green Hydra (Schummer *et al.*, 1992). What is striking is that although all the vertebrates have more than one copy, the non-vertebrates do not (Holland, 1991).

The function of the *msh* gene in *Drosophila* is not known. The expression patterns of the *Msx* genes in mice and chickens imply a role in development in various disparate parts of the body. *In situ* studies of the *Msx* genes reveal complex and dynamic expression patterns which are overlapping and partly complementary.

Expression of the *Msx* genes in vertebrates:

This is a summary of published and unpublished data for expression in mice and chickens. Most statements about embryonic age refer to the mouse. At equivalent developmental stages, both animals show very similar expression patterns.

Early expression:

Msx1 message detected in extraembryonic cell mass from E6.5; also in the amnion (E7-E16) and the allantois (E7.5) (Lyons *et al.*, 1991, Suzuki *et al.*, 1991).

At gastrulation, *Msx1* and *Msx2* are expressed in the primitive streak. After gastrulation, both genes are expressed in all three embryonic layers (Davidson, pers. comm.) - laterally in the neural folds, also in

somatic and splanchnopleuric mesoderm (Suzuki *et al.*, 1991). The mesodermal boundary of *Msx1* expression lies more medially than *Msx2* (Davidson and Hill, 1992, Yokouchi *et al.*, 1991). *Msx1*, at least, continues to express in the mesoderm of the ventral tail bud, a remnant of the primitive streak, until E10.5 (Lyons *et al.*, 1991).

CNS and neural crest:

Msx3 is reported in the dorsal neural tube of the hindbrain and trunk from E7.5-E10 (Grindley, pers. comm.).

E7.5-E8: *Msx1* and *Msx2* are expressed laterally in the neural folds in regions which, after closure, become the dorsal neural tube and brain (Davidson, pers. comm; Suzuki *et al.*, 1991).

The genes are expressed all along head and trunk CNS, except in the hindbrain where *Msx1* and *Msx2* are initially expressed only in rhombomeres 3 and 5 (*Msx3* is upregulated in r2, r4 and r6 (Shimeld, pers. comm.)). This pattern is transitory - all three genes are then expressed dorsally in all rhombomeres.

Expression in the roof of the neural tube and brain is maintained until E12.5. By E11.5, the dorsal epithelial layer of cells at the roof of the forebrain express *Msx2* only (Nishikawa *et al.*, 1994).

Migrating neural crest: Neural crest migrates between E7.5, (6 somite-stage), and E9 in the head, between E8 and E10 in the trunk (Serbedzija *et al.*, 1990, 1992). Hence all migrating neural crest cells must express *Msx1* and *Msx2* when they delaminate. In the trunk, expression is maintained very transiently after leaving the neural tube and none of the neural crest-derived structures (e.g. dorsal root ganglia, differentiated Schwann cells and melanocytes etc) express the genes (pers. obs.; Yokouchi *et al.*, 1991).

Expression is maintained longer in cranial neural crest, and at E10-E11, much of the dorsal neural crest-derived mesoderm of the head is positive for *Msx1* and *Msx2*. (Lyons *et al.*, 1991; Hill *et al.*, 1989; Mackenzie *et al.*, 1992)

Msx1 and *Msx2* are also detected in the meninges and choroid plexus from E12-E17. *Msx2* is much more strongly expressed than *Msx1* at these sites (Nishikawa *et al.*, 1994; Lyons *et al.*, 1991).

The face:

From E9-E11, *Msx1* and *Msx2* are expressed in the neural crest-derived mesenchyme and overlying epithelium of all the branchial arches (Mackenzie *et al.*, 1991, 1992; Suzuki *et al.*, 1991; Hill *et al.*, 1989).

Both maxillary and mandibular processes label intensely, as do the fronto-nasal and lateral-nasal epithelium and mesenchyme. In all cases, the mesenchymal domain of *Msx2* lies within that of *Msx1* (Nishikawa *et al.*, 1994).

Expression within the tissues of the face subsequently becomes more localised (Mackenzie *et al.*, 1992).

Tooth development: E10.5 - *Msx2* starts to localise to the mesenchyme of the presumptive molars. At E11.5 *Msx2* expression switches to the epithelium of the dental placodes, and at E12 is found in the invaginating dental lamina. At E13-14 it localises to the enamel septum, knot and internal enamel epithelium, before appearing in the mesenchymally-derived differentiating odontoblasts at E17 (Mackenzie *et al.*, 1992). Expression of *Msx2* in the dental follicles and papilla is very weak, in contrast to *Msx1*, which is expressed strongly only in these structures (Mackenzie *et al.*, 1991, 1992).

Also, in the head between E12 and E17, *Msx1*, and *Msx2* are expressed in the cells adjacent to Meckel's cartilage, in the whisker follicles, Rathke's pouch, thyroid duct, branchial clefts and Jacobson's organs (Lyons *et al.* 1991; Mackenzie *et al.*, 1991, 1992).

Musculature:

In spite of the name, *Msx* genes are characteristically not expressed in vertebrate skeletal muscle. *Msx1*, but not *Msx2*, is expressed in determined myogenic cells of the ventrolateral dermamyotome at the level of the limbs at E9-E10 (pers. obs.). Some of the somite-derived muscles of the tongue and cheeks may express *Msx1* at E12-14 (Davidson, pers comm.).

Eyes and ears:

At E9.5, *Msx2* is expressed in the surface epithelium of the eye, (including lens placode), and the out-pocketing neural epithelium which will become the optic cup (Monaghan *et al.*, 1991). At E10 it is expressed in the inner (neural retina) layer dorsally in the optic cup and in the invaginating lens epithelium. It becomes localised, at E11 and E12, to the corneal epithelium and becomes more distally restricted in the optic cup so that by E13.5, expression is restricted to the prospective ciliary body region near the distal rim of the cup (Holme, pers. comm.).

Msx1 is expressed in the periocular mesenchyme at E9-10, dorsally in the neural retina from E11.5 in a domain which overlaps with *Msx2*, but is more distally restricted; from E12-E17, it is expressed in the differentiating ciliary body. *Msx* expression precedes morphological differentiation of the ciliary body.



Data from the developing ear is incomplete. *Msx1* is detected in the dorsal third of the invaginating otic vesicle from E9, a pattern which is maintained when the vesicle closes and which is mirrored by *Msx2*. (Lyons, 1991; pers. obs.). Subsequently, *Msx1* is expressed in the mesenchyme of the developing pinna, whereas *Msx2* is expressed in the epithelium only.

Heart:

Expression of *Msx1* and *Msx2* is non-overlapping. *Msx1* is expressed in a strip of cells in the cardiac endothelium at E8-10. Subsequently, it is expressed in the delaminating cells of the atrioventricular junction and outflow tract which are undergoing a mesothelial transition to form the endocardial cushions. Later it localises to the atrio-ventricular and semi-lunar valves.

Msx2 is restricted to a subset of differentiating myocardial cells which will contribute to the cardiac conduction system (Chan-Thomas *et al.*, 1993; Suzuki *et al.*, 1991).

Limbs:

Msx1 and *Msx2* are expressed in the marginal mesenchyme, including the Progress Zone, and the AER of the limb bud from E9-E12, after which they are expressed interdigitally and in the perichondrium of the digits (Hill *et al.*, 1989; Robert *et al.*, 1989, Suzuki *et al.*, 1991, Yokouchi *et al.*, 1991, Coelho *et al.*, 1991a; 1992a, Nohno *et al.*, 1992)). I looked very carefully at *Msx* genes in the limb, and this analysis is described in Chapter 3.

The urinogenital system:

Not well defined. *Msx1* is expressed in the mesoderm of the genital eminence at E11.5, and subsequently, at E15, in the genital tubercle (Lyons *et al.*, 1991).

In females, it is expressed throughout the Mullerian duct epithelium perinatally, but expression is soon restricted to the uterine epithelium and is maintained throughout life. *Msx1* is expressed in the uterine wall of pregnant mice (Pavlova *et al.*, 1994).

Other domains of *Msx* gene expression after birth:

Msx2 is expressed in cells of the membranous bones of the skull at the calvaria, and in adjacent mesenchymal cells, during late embryonic and early post-natal mouse development (Jabs *et al.*, 1993).

Msx expression elsewhere in adults has not been properly studied. Dermal matrix cells in hair follicles are known to express *Msx1* and *Msx2* (Wang and Sassoon, 1995). *Msx2* is expressed in mature osteoblasts (Hodgkinson *et al.*, 1993). *Msx1* and *Msx3* are expressed in the stroma of mammary glands of pregnant mice, whereas *Msx2* is expressed in the nipple epithelium (Holland, pers. comm).

Significance of domains of *Msx* expression:

Many of the sites of *Msx* expression are coincident with the areas of increased vertebrate bodily complexity (compared to the invertebrates) that were described earlier. This is of course consistent with the theory of 'new genes for new jobs', also outlined earlier and is reinforced by the fact that the single *Branchiostoma msh* gene (Holland, 1991) is expressed in embryos in the dorsal nerve cord only. This ancestral site of expression appears to be retained by all three *Msx* genes. However, whilst preliminary data shows that *Msx3* is expressed only in the nerve cord during embryogenesis, the other two genes are expressed in the additional vertebrate-specific regions as well.

Msx genes are expressed in many tissues, primarily in cells which are not terminally differentiated (Hill *et al.*, 1989). They are hence implicated in having roles in the differentiation process. Their expression patterns, in specific, separated domains, is somewhat different from the broad-banded *Hox* gene expression patterns, suggesting that they are several steps away from the primary pattern-forming mechanisms. They may offer an opportunity to study the genetic pathways which mediate between *Hox* gene control of the differentiation process, and the resulting cell-fate determination.

In some areas of the body - the heart and teeth - the expression domains of *Msx1* and *Msx2* are very different; in others they overlap - limbs, dorsal neural tube and brain, facial processes - often with the expression domain of one gene contained within that of the other. Different-but-overlapping expression domains may indicate that the different *Msx* genes have different roles during organogenesis, as was recently proposed for *msha-d* in *Danio*; these genes show expression in different subsets of tissues during fin growth and regeneration (Akimanto *et al.*, 1995) and in the inner ear (Egger *et al.*, 1992).

Many of the sites of *Msx* gene expression are also sites of tissue-tissue, especially mesenchymal-epithelial, interactions which cause proliferation or differentiation in the tissues involved (Lyons *et al.*, 1991). These sites include limb buds, the facial primordia (Wedden, 1987), the teeth, the genital eminence, the tail

ridge, the perichondrium of the digits, the genital tubercle, the heart, the membranous bones of the skull, the hair follicles and the otic vesicle.

Study of *Msx* genes may define the genetic processes which are occurring during these interactions, and define the cascades of gene expression which refine the limb patterning system.

In different areas of the body, *Msx* gene expression has been shown to be maintained by tissue interactions leading cells into several developmental fates.

The limb bud has been the best studied of these systems.

Chicken or mouse limb mesenchyme, when cultured in the absence of ectoderm, downregulates *Msx1* expression (Watanabe and Ide, 1993; Wang and Sassoon, 1995). This suggests that *Msx1* expression is maintained by the overlying ectoderm.

In the chicken *limbless* mutation the limb bud shows normal morphology and *Msx* expression until stage 19, when the AER fails to form (Carrington and Fallon, 1988). *Msx2* is not upregulated in distal epithelium, and because the AER is not functional, cells in the Progress Zone fail to proliferate. They lose *Msx1* expression, and then die (Coelho *et al.*, 1991). Grafting a wild type epithelium onto a *limbless* mutant wing rescues proliferation and *Msx1* expression in the Progress Zone. Hence *Msx1* and *Msx2* expression in the AER and Progress Zone are dependent on the interaction between the tissues (Robert *et al.*, 1991).

These observations are confirmed by removal of the ridge, which leads to loss of *Msx1* expression in most of the subjacent mesenchyme of the Progress Zone. Cells stop proliferating and die (Ros *et al.*, 1992).

In other mutations, the two way interaction between an AER and the progress zone is shadowed by altered expression of the *Msx* genes. In the polydactylous mutations, *diplopodia*⁵ and *talpid*², which have an extended ridge and broadened Progress Zone, the expression domains of *Msx2* in the ridge and *Msx1* in the mesenchyme are extended to match (Coelho *et al.*, 1992a,b, 1993b). In the creation of a phenocopy of the *eudiplopodia* mutation, a second AER was grafted dorsally on the wing at stage 24, with a concomitant induction of *Msx1* in the subjacent mesenchyme (Robert *et al.*, 1991).

The AER can maintain *Msx1* expression in limb mesenchyme cells in culture (Coelho *et al.*, 1993). There is evidence that it can also induce it *de novo*. Davidson *et al.* (1991) showed that if proximal (non-expressing) mesenchyme was taken from an 11.5d mouse hind-limb and grafted into the distal mesenchyme of a stage 20 chicken wing, immediately underneath the AER, the mouse mesenchyme

induced *Msx1* strongly and quickly (within 5 hours); *Msx2* was also induced in the distal-most cells. Distal, expressing, mesenchyme, if grafted proximally into a wing, turned off. In all cases, the mouse graft only expressed *Msx1* and *Msx2* in those areas of the chicken wing which were expressing the genes.

These experiments showed that *Msx* gene expression in limb mesenchyme was controlled by position-specific signals, one of which is released by the AER. They also demonstrate that these signals are compatible between mice and chickens, and that non-expressing tissue (which other experiments showed to be developmentally plastic) was capable of reinitiating gene expression.

These conclusions were to be fundamental to the experiments I was to perform.

Other genes whose expression in the limb mesenchyme is maintained by the AER include *connexin43* (Green *et al.*, 1994) and *PRX-1* (Nohno *et al.*, 1993). Both of these genes show expression patterns like *Msx1* (pers. obs. - see Chapter 3) and may therefore be involved in the same developmental pathways). Wang and Sassoon (1995) report very rapid downregulation of *Msx1* in dissociated limb mesenchyme cells; mesenchymal cell-cell interactions are hence implicated in the maintenance of *Msx1*, so the gap-junction protein Cx43 is potentially important for these interactions.

In a separate set of experiments, Brown *et al.* (1993) demonstrated a similar situation in the face. In the facial primordia, the epithelium has been shown to promote proliferation in the mesenchyme of the facial processes, as in the limb. *Msx1* and *Msx2* are expressed in the facial epithelia and distal mesenchyme (see earlier). Brown *et al.* showed that expressing or non-expressing mesenchyme from any of the facial primordia, when grafted distally into the mandibular mesenchyme of the chick, expressed *Msx1* after overnight culture, but not if grafted proximally. Face mesenchyme could also turn on if grafted distally in the limb, but the reciprocal graft did not work.

Jowett *et al.* (1993) showed that maintenance of *Msx1* and *Msx2* in the teeth is dependent on tissue-tissue interaction. *Msx1* expression in the mesenchyme needs a non-specific (dental or non-dental) epithelial signal; *Msx2* expression in epithelium or mesenchyme requires reciprocal interactions between specialised dental cell populations.

Msx2 expression in the tooth parallels very closely that of *Bone Morphogenetic Protein-4* (*Bmp4*) (Heikenheimo, 1994) and is a marker for cells with the ability to respond to signals passing between the epithelium and mesenchyme. Application of ectopic BMP4 was shown to be able to induce *Msx1* and *Msx2* in dental mesenchyme (Vainio *et al.*, 1993).

Differentiation of neural crest-derived mesenchyme of the mandible into membranous bone requires a signal from the mandibular epithelium. Takahashi *et al.* (1992) showed in quails that expression of *Msx2* in the mesenchyme correlates with the ability to respond to the epithelial signal which initiates differentiation, and that heterotypic epithelia will suffice. Takahashi also showed that expression in the roof plate of the neural tube depends on an interaction between the dorsal neural tube and the superficial epithelium, and that expression of *Msx2* in the neural crest-derived mesenchyme dorsal to the neural tube correlates with the ability to differentiate into the spinal processes of the vertebrae.

Pavlova *et al.* (1994) showed that maintenance of expression of *Msx1* in the adult uterine epithelium is maintained by the mesenchyme of the uterine wall and correlates with *Wnt5a* expression in the mesenchyme. Maintenance of *Msx1* in the adult uterus, when it is downregulated in the vagina and cervix, correlates with the ability to undergo the large changes in uterus morphology during pregnancy.

Msx1 and (especially) *Msx2*, expression in rhombomeres 3 and 5 precedes the apoptotic cell death which kills the neural crest cells which would otherwise migrate from these rhombomeres. The even-numbered rhombomeres have been shown to maintain *Msx2* expression and cause cell death in r3 and r5 (Graham *et al.*, 1993). In culture, application of BMP4 to isolated rhombomeres induces *Msx2* and initiates apoptosis.

Msx2 is expressed in mature osteoblasts. It can be shown in culture to be induced in osteoblastic cell lines by vitamin D₃, which is known to promote proliferation at the expense of differentiation (Hodgkinson *et al.*, 1993). *Msx2* has been shown to be over-expressed in some human tumour cell lines (Suzuki *et al.*, 1993)). This may or may not be physiologically significant, but it does reinforce the role of *Msx* genes in the making of developmental decisions; a mistake in developmental regulation can correlate with a mistake in *Msx* expression.

Msx genes may enable the investigator to study the genetic basis of tissue-tissue interactions and they may also be part of the genetic basis of developmental plasticity and the ability to redifferentiate. Gardiner *et al.*, (1995) have shown that the limb regeneration blastema of Axolotl expresses *Msx2* (and several *Hox* genes). Reginelli *et al.* (1995), have shown that mouse embryos can regenerate an amputated digit tip, providing only the distal, *Msx*-expressing, tip is cut off. For regeneration, the cells at the amputation site must be expressing *Msx1*, but not necessarily *Msx2*; the cells of the resulting blastema express both genes. This is true also for neonates and adults, where the tip can regenerate only if the cut-site passes through the *Msx1* and *Msx2* expressing cells of the nail bed.

There are lots of correlations between *Msx* gene expression and cellular processes. Surprisingly, very few of these have been exhaustively studied. Even in the limb, an extensively studied developmental system, there has been no explicit attempt to confirm or refute that *Msx* expression domains completely overlap such areas as the ZPA, the apoptotic zones and the Progress Zone throughout development. This was to be one area I would investigate.

Sometimes, correlations are not enough. We need to see how direct manipulations of the *Msx* genes affect cellular processes. Some experimental manipulations have been performed and these, together with future directions for work, are reviewed in the next section.

Strategies for the analysis of *Msx* control and function:

I wanted to perform some experiments to investigate the control and function of the *Msx* genes during limb development, a process in which there is evidence that the *Msx* genes may be involved, in respect of both their expression patterns, changes of expression under experimental manipulation and in mutant animals with abnormal limbs. These studies would be important in their own right, but it was hoped that it would be possible to infer some answers to the questions posed about the control and function of the homeobox genes in general, and about the role of this class of homeobox genes during vertebrate evolution

It is difficult to know whether, in mutant or experimentally-manipulated animals, the perturbation of *Msx* expression is causal or consequential to the phenotypic effects described. There are no known abnormal limb phenotypes which are provenly caused by a mutation in an *Msx* gene (Robert *et al.*, 1994); in common with many other homeobox-containing genes it appears therefore that animals which are heterozygous for a loss of function mutation are phenotypically normal - there is haplo-sufficiency.

A human family with inherited Boston-type craniosynostosis (the bones of the cranium fuse prematurely) have been found to be carrying a non-silent mutation in the *MSX2* homeobox which segregates absolutely with the disease (Jabs *et al.*, 1993). This was the first direct evidence that the *Msx* genes are really doing something. The members of this family do show some limb abnormalities - one of them has an ectopic thumb and several show shortened phalanges (It should perhaps be remembered that limb abnormalities are not uncommon, and there is no reason to assume that the ectopic thumb is related to the *MSX2* malfunction). A phenocopy of this defect has been produced by introducing the same mutation into transgenic mice (Liu *et al.*, 1995).

Considering the paucity of spontaneous mutations in the *Msx* genes which may give clues to their functions, a feasible way of studying them is by reverse genetics - creating mutations by manipulation of the *Msx* genes. The technology is available to knock out any of the genes in mice by homologous recombination in ES cells. This has been done for *Msx1* (Satokata and Mass, 1994) and *Msx2* (unpublished) separately and surprisingly the homozygous null is not an early lethal state.

I had presumed that because *Msx* genes were expressed at gastrulation, the knockout mice would die at this stage. The parallel example is that of the *Fgf4* knockout mice. *Fgf4* is also needed for limb development, but the knockout is not useful in analysing its role because it dies soon after implantation

when the ICM fails to proliferate. (Feldman *et al.*, 1995). *Evx1* is another example of a limb gene whose knockouts fail to complete gastrulation. (Spyropoulos and Cappechi, 1994).

The *Msx1* knockouts do not fully develop teeth and, probably as a result of this (Satokata and Maas, 1994; Ferguson, 1994), the palatal shelves fail to fuse, leading to death soon after birth - they cannot suckle properly and their stomachs fill with air. They also exhibit slight deformations in the membranous alveolar, parietal, maxillary and mandibular bones of the skull and lack a process of the maleus bones - all the abnormalities are in first arch derivatives. *Msx2* knockouts (unpublished) are reported to have CNS defects.

There are many sites of *Msx1* expression which do not show abnormalities in the knockout mice (limbs, eyes, heart etc.). *Msx1* and *Msx2* show a large degree of overlap over much of their expression domains and it now seems likely that there is some functional redundancy - where one *Msx* gene is missing, another one can perform its job adequately enough in many body areas to avoid a phenotype. (This would be similar to the situation seen for the *Engrailed* genes *En1* and *En2*, where the *En2* knockout has no obvious phenotype, even though the *En1/En2* double knockout is more severe than the *En1* knockout alone). In this respect it is interesting to note that the teeth and the heart are two areas where *Msx1* and *Msx2* overlap very little. The double knockout would, of course, be informative in characterising the degree of functional redundancy between the genes.

No limb phenotype was reported for the *Msx1* knockout mice. This is possibly because of functional redundancy between the *Msx* genes in the limb; the expression pattern of *Msx2* in the mouse limb was incompletely known, so I analysed a series of whole-mount *in situ* hybridisations of *Msx1* and *Msx2* in mice to investigate the degree to which they are co-expressed.

An investigation into the function of the *Msx* genes attempts to define what jobs *Msx*-expressing cells can do that non-expressing cells do not. One way to ask this question is to mis-express the functional protein, either in cultured cells or in the developing embryo. Mis-expression of *Msx* genes in cells in culture has provided information about their possible roles (Song *et al.*, 1992). If it were possible to create a mutation which affected the expression of the *Msx* genes only in body areas which would not severely affect the viability of the embryo then we may see a phenotype which could tell us something about the function of the genes. This is what Mother Nature appears to have done with *MSX2* in the craniosynostosis family. The eye, the limbs and the teeth are obvious targets in which to alter gene expression, since their loss need

not result in immediate embryonic death. Bearing in mind the history of experimental embryology behind it, the limb would be a prime site for this sort of investigation.

Unfortunately, at present not enough is known about control of *Msx* genes to be able to manipulate them with the *finesse* required. One way to find out more about the promoter is to do a functional dissection - to cut bits out and see which aspects of gene expression are upset.

Functional dissections of eukaryotic promoters:

Functional dissections of many promoters have been achieved by using the *lacZ* coding sequence, in frame with the promoter sequences of choice, to look at expression in transgenic animals.

A reporter gene such as *lacZ* is used because:

- (i) it is easy to check for gene expression without having to wait for *in situs* (MacGregor *et al.*, 1989);
- (ii) the endogenous genes are unaffected and (apart from ectopic expression of β -galactosidase!) all embryos are wild-type.

This was performed successfully on the *ftz* and *eve* genes of *Drosophila* and subsequently with several vertebrate developmentally-regulated genes.

In the case of *fushi tarazu*, 6kb of *ftz* promoter was linked in frame with *lacZ* coding sequence and introduced into flies using a P element (Hiromi and Gehring, 1985).

It was found that transgenic flies expressed β -gal in a pattern which exactly matched that of endogenous *ftz* at gastrulation (the seven stripes) and later in the ventral nervous system. In parallel experiments, transgenic flies which expressed *lacZ* under the control of only 2.4 kb of *ftz* promoter showed only very weak expression (although the reporter was expressed in all the right places). Flies which had only 600bp of *ftz* promoter showed the seven stripes at gastrulation but had no expression in the ventral nervous system. Flies with less than 600 bp of promoter lost all expression of the reporter. On the basis of this data it was possible to subdivide the *ftz* promoter into areas which contained an upstream enhancer, an area with a neurogenic element, and a 'zebra' element which is responsible for the stripes.

In mammals, specific enhancer elements have been found upstream of homeobox and non-homeobox genes by using subsets of their promoters to drive *lacZ* expression in the embryo.

Some of the enhancer elements are tissue-specific, some are responsible more for general position-specific expression, e.g.

Studer *et al.* (1994) found a 322bp element necessary to drive r4-specific expression of *Hoxb1*. Schlaeger *et al.* (1995) isolated 1.2kb of promoter specific to vascular endothelial cell expression. Tuggle *et al.* (1995) - a 604bp enhancer in the promoter of *Hoxb5* was necessary to drive *lacZ* expression in the dorsal cells of the branchial region, and 2.3kb of *Hoxd4* 5' region necessary for expression in the upper cervical spinal cord. Knittel *et al.* (1995), found a 470bp control element, 1.6 kb upstream of *Hoxa7*, sufficient to establish the anterior boundary of expression. Schughart *et al.* (1991) discovered limb-specific elements 1.2-3.6kb upstream of *Hoxb6*. Echelard *et al.*, (1994) - a 5.5kb *cis*-acting 3' sequence can drive the complete *Wnt1* expression pattern during embryonic development. (etc. e.g. Bierberich *et al.* (1990), Gerard *et al.* (1991)).

The 439bp element responsible for driving *Msx2* expression in the AER was found this way (Liu *et al.*, 1994).

The technique showed firstly that eukaryotic promoters are in general accessible to such dissection (in terms of having tissue-specific and position-specific enhancer elements) and secondly that the broad elements of expression are reproducible when the transgene is at a different genomic site from that of the endogenous gene.

A similar functional dissection of the promoter of *Msx1* would be desirable. By linking the promoter of *Msx1* to the *lacZ* coding sequence it should be possible to produce transgenic mice expressing the reporter gene in a pattern which reflects expression of the endogenous gene. Then, as in the experiments described and referred to above, mice could be made carrying reporters which do not have all of the promoter to see if any aspects of gene expression go missing or if any new areas of expression appear.

There are disadvantages with this technique, in that transgenes are susceptible to position effects due to their sites of insertion in the genome. This may be due to either being influenced by the enhancers of genes surrounding the insertion site, or being silenced by a permanent chromatin conformational change. This latter problem is particularly relevant to transgenes which have passed through the germ line. (Allen *et al.*, 1988)

Careful analysis of expression in several transgenic founders is necessary to separate genuine expression from position effect (e.g. Charite *et al.*, 1994); nevertheless, had a transgenic facility been available in 1991, this would have been my method of choice for investigating control of *Msx1*. Transgenic mice

were available which had *lacZ* in frame with 4.7kb of *Msx1* 5' sequence, and I shall return to these mice in Chapter 5.

A potentially quicker method to get a degree of functional dissection of the *Msx1* promoter would be to introduce the reporter construct into a subset of cells in the embryo. It is known (see earlier) that *Msx1* expression in the Progress Zone region of the limb bud is under position-specific control. By introducing various reporter constructs (*lacZ* coding sequence on *Msx1* 5' promoter regions) into cells, and then introducing these cells into the limb, it would hopefully be possible to determine

- (i) a promoter which is turned on only in the same regions at the same times as the endogenous gene (the whole promoter);
- (ii) promoter regions which do not contain the whole *Msx1* promoter and where the reporter is turned on in regions of the limb where the endogenous gene is not expressed, or which is not turned on where the endogenous gene is expressed.

In this way it may be possible to define regions of the *Msx1* promoter which are necessary for correct position and time-dependent expression in the limb. In the ideal world, the aims of these experiments would be to see what the effect is when the endogenous *Msx1* gene is expressed on these altered promoters - either by creating transgenic mice in which the endogenous gene had one of these altered promoters (to see what the phenotype is), or alternatively to introduce cells expressing the endogenous gene under these altered promoters into an otherwise normal embryo (to see whether the altered cells showed inappropriate cell fates).

This approach would also be appropriate to examining the control of gene expression in any area of the body which is accessible to grafting. One of its advantages is that it is possible to avoid confusion caused by position effects, and that it avoids the problems caused by taking a transgene through the germline.

There are other approaches by which it would be possible to get inappropriate expression of *Msx* genes in a restricted, non-lethal, area of the body (such as the limb). Disabled retroviral vectors can be injected into the limb to deliver an altered copy of the gene to a subset of cells in the limb (this was done recently in the investigation of the role of *Shh* (Riddle *et al.*, 1993)). If the vector also carries a marker (such as *lacZ*) so that infected cells can be recognised, then the effect on cell fate can be determined. One potential experiment would be to infect cells with a copy of *Msx1* on a constitutive promoter and see

whether infected cells which were expressing *Msx1* inappropriately showed an altered or restricted cell fate. The technology to perform this experiment is available.

The 'functional dissection' approach is an orderly means of identifying promoters upon which it is possible to mis-express *Msx* genes in transgenic mice and attempt to get a phenotype. Many genes are expressed in similar parts of the body to the *Msx* genes e.g. *Pax6* (in the eyes) (Li *et al.*, 1991), *Wnt1* (developing neural tube), *Wnt5a* (developing limb bud), *Fgf4* (AER), *Krox20* (rhombomeres 3 and 5) (see earlier). In particular, the expression patterns of *Msx1* and *Msx2* overlap for large areas. It would be possible to create a construct which expresses *Msx1* under the control of a promoter of one of these genes. If this construct were then introduced into transgenic mice it is conceivable that the perturbation of *Msx* gene expression may not be great enough to kill the embryo before an informative phenotype is seen. The experiment which I started, aiming to express *Msx2* under the control of 4.7kb of *Msx1* 5' sequence in transgenic mice, was never finished; it is undoubtedly a worthwhile experiment.

So, although little is proven about the function of *Msx* genes apart from that they are likely to be important during vertebrate embryonic development and that their control is likely to be complex, they are not completely refractory to experimental investigation. They offer opportunities to look at the processes of differentiation, tissue interactions and maintenance of developmental plasticity. They may be one of the links between the *Hox* genes and structural morphogenesis. With a little imagination it is possible to develop a strategy by which to attack them and to see where they stand in the story of the first few days of an embryo's life.

Aims of the thesis

Given that the eventual aim of any study of *Msx* genes is to find out what the genes are doing, there have been certain key barriers to progress.

1. It will be obvious from the descriptions on pages 49-53 that published accounts of *Msx* gene expression have been incomplete, imprecise or over-summarised. To be able to relate any experimental investigations to gene function it is fundamental to know exactly where the genes are normally active in the embryo. Chapter Three will address the expression of *Msx1* and *Msx2* in the limb buds of mice and chickens; the discussion in Chapter Eight will relate these expression patterns to known developmental domains and attempt to define possible roles for the genes.
2. Direct sequencing of the promoter has proven not to be the best way of analysing control of *Msx1*. The experiments of Davidson *et al.* (1991) however provided a method of investigating the extrinsic factors

which control the genes. Grafting experiments allow one to look at the responsive potential of a promoter in a specific tissue interaction, but there is also a need to relate specific elements of the inductive environment (e.g. the factors released by the AER) to specific domains in the promoter. This would, in future experiments, enable one to manipulate expression of the endogenous gene in a defined situation (such as the AER/Progress Zone interaction) to study gene function without causing pleiotropic lethal effects in other areas of the body. Chapter Four will describe the first series of grafting experiments which attempted a dissection of the *Msx1* promoter, using reporter constructs (described in Chapter Two) which express *lacZ* under the control of *Msx1* regulatory sequences. For reasons described in the text, these experiments were performed using 10T¹/₂ cells to host the reporters, and these cells were not shown to induce *lacZ* in response to the AER signal. Chapter Five describes lines of transgenic mice which express *lacZ* under the control of 4.7kb of *Msx1* 5' sequence, and compares embryonic *lacZ* expression with that of endogenous *Msx1*. Using these transgenic mice as a source of limb mesenchyme which contains a reporter construct, Chapter Six goes on to describe a further series of grafting experiments which define the responsiveness of the transgenic promoter to inductive signals during embryogenesis. Chapter Eight will summarise what has been learnt from the behaviour of grafted cells which contain the reporters, and suggest implications for the control of the endogenous *Msx1*.

3. The power of grafting experiments (that they allow one to modulate gene expression without knowing what is happening at the molecular level to the promoter) is also their weakness. For example, as noted by the authors of Cohn *et al.* (1995), the induction of ectopic limbs by application of Fgfs to the flank (page 47), may explain why nasal placodes (which express *Fgf8*) can also induce ectopic limbs when grafted to the flank - one always wants a molecular explanation for the phenomena revealed by grafting technology. There is always a need to get back to the molecular level to be able to define which genes are immediately upstream and downstream of the *Msx* genes. Chapter Seven establishes *Bmp4* as a molecule which can signal to cells to induce expression of *Msx* genes, but also shows that it does not affect expression of the transgene, potentially explaining the apparent inability to induce *lacZ* in distal limb grafts.

4. Because the burden of 'evolutionary significance' has been placed on the *Msx* genes it is necessary, in the wider context, to link experiments to the plausibility of theories of vertebrate evolution, in relation to the evolution of vertebrate-specific tissue and structures. Chapter Eight relates the expression of the transgene to possible genomic changes during evolution and, in conjunction with knowledge of the *Bmp4/Msx* interaction, suggests how divergence of paralogous gene families may have influenced the evolution of the vertebrate body plan.

CHAPTER TWO

MATERIALS AND METHODS

PREVIOUS WORK

MATERIALS AND METHODS

The cell lines described in this chapter were derived from the primary tumor of a patient with a histologically confirmed diagnosis of squamous cell carcinoma of the head and neck. The patient was a 65-year-old male who presented with a large, ulcerated mass in the right oral cavity. The tumor was resected and the patient underwent postoperative radiation therapy. The cell lines were established from the primary tumor and the postoperative radiation therapy specimen.

Cell Culture

The cells were derived from primary tumor and postoperative radiation therapy specimen. The cells were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells were grown to confluence in 25 cm² flasks and then harvested by trypsinization. The cells were then seeded into 96-well plates at a density of 10,000 cells per well. The cells were grown for 24 hours before the start of the experiment.

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CHAPTER TWO

MATERIALS AND METHODS

PREVIOUS WORK:

I have been fortunate in having a ready-made supply of raw material upon which to work. This meant that I needed to produce no new cell lines or reporter constructs to make a start on my experiments. Even the transgenic mice were available for use. Briefly I present here an introduction to the cells, plasmids and transgenes I used, with a vote of thanks to the people who made them.

CH3 10T^{1/2} cells:

These cells were derived from embryos of the inbred mouse CH3 line at the McArdle Laboratory for Cancer Research (Reznikoff *et al.*, 1973). They are an immortal, non-tumourigenic cell line, highly sensitive to post-confluence inhibition of cell division. They show many phenotypic and biochemical similarities to the 3T3 cell lines isolated from BALB/c, NIH and Swiss mice.

The cells have a characteristic rounded morphology and form flat even, fibroblastic, monolayers at confluence, when most cells arrest in G1.

They are hypertetraploid with 81 chromosomes (2N=40 in mouse) and a generation time of 15.5 hours at 37°C in medium containing 10% FCS. The average volume of the cells is 1730mm³ and confluence density is 29000-38000 cells per square centimetre at confluence.

Because of their genomic stability and non-tumourogenicity in culture, they have been used in studies of oncogenic transformation by chemical and physical mutagens (e.g. Terzaghi and Little 1975).

They can differentiate into myogenic, chondrogenic and adipocytic phenotypes under experimental manipulation - this will be explained more fully in Chapters Four and Eight.

H3M cells:

An unpublished cell line made by Spike Clay at the MRC Human Genetics Unit. Derived by spontaneous transformation of E9 neural tube cultures, grown in the presence of LIF. They may be neural crest derived, although they have never been tested for expression of neural markers. They have epithelial morphology and show contact-mediated growth inhibition.

Plasmids:

The reporter constructs, pH7lacT and pH7lacΔ3':

The plasmids pH7lacT and pH7lacΔ3' were made by Bob Hill in 1989.

An *Nco*I site was introduced between the *Kpn*I and *Hind*III sites in the 5' coding sequence of *lacZ* in pCH110. The coding sequence, with its internal *Nco*I site, was then cloned as a *Sau*I//*Bam*HI fragment, in frame, into the *Sau*I//*Bam*HI sites in the polylinker of pGEMEX-1, where it could be transcribed from the T7 promoter to ensure that it retained activity. The 3' *Bam*HI site was 3' to the stop codon of *lacZ*, but the 5' end of the ORF was truncated.

A 4.7kb *Nco*I fragment was isolated from mouse genomic DNA, containing *Msx1* 5' sequences including the immediate proximal promoter sequences, presumptive transcription start site (from published cDNA), the published ATG start codon and the first 127bp of coding sequence. (1721bp between the 3' *Nco*I site and an upstream *Eco*R1 site has been sequenced (Townley (1995))). This fragment was cloned into the *Nco*I site created in *lacZ*. The *lacZ* ORF was in frame and hence a chimaeric *Msx1*/β-Gal protein would be produced under control of *Msx1* promoter sequences.

pH7lacT and pH7lacΔ3' differed only in the sequences which were added 3' of the stop codon of *lacZ*

pH7lacΔ3';

SV40 poly-A addition and termination signals were then blunt-end ligated into the 3' *Bam*HI site. This ensures efficient translation of the mRNA (Fig. 2.1A).

pH7lacT;

A *Sac*II//*Not*I fragment was isolated from the *Msx1* genomic DNA which starts in the *Msx1* coding region, has the *Msx1* intron, followed by the homeobox, the stop codon and ~3kb of 3' regions which contain the *Msx1* 3'UTR, processing and termination signals. The 5' (*Sac*II end) was blunt end ligated to the *Bam*HI site at the 3' end of *lacZ*, with the *Not*I 3' end being cloned into a *Not*I site in the plasmid. None of the *Msx1* coding sequence is translated because of a stop codon at the 3' end of *lacZ* (Fig. 2.1B).

Plasmids used for the constitutive expression of *lacZ*:

1. pCMV β :

(Fig.2.1C)

This plasmid was designed to work at high levels in a wide variety of cell types, which it appears to do (Grant MacGregor, pers.comm.).

The promoter is the human cytomegalovirus immediate early promoter/enhancer. This is in frame with the β -gal coding sequence and there is an RNA-splice signal and a polyadenylation signal to ensure adequate nuclear export and messenger processing. The reporter gene was cloned into pUC19.

2. pSV β :

(Fig. 2.1D)

Designed to work in COS cells, I found that expression in other cell lines was as good as that of pCMV β . It is similar to pCMV β , but the SV40 early promoter/origin is used to drive expression of *lacZ*.

COS cells carry the SV40 large T antigen gene, constitutively expressed at high levels, and hence will amplify (~10000x) introduced pSV β .

Transgenic Mice:

A number of lines of transgenic mice were created prior to my arrival, in collaboration with Austin Smith and Rosa Beddington at the Centre for Genome Research, Edinburgh. The 8.5kb *Sfi1/Not1* fragment from pH7lac Δ 3' was injected into the male pronucleus of fertilised mouse oocytes, which were transferred to pseudopregnant females. Resulting births were allowed to wean, tail-tipped, and genomic DNA thus prepared was Southern blotted. Probing with ³²P-labelled probe prepared from the *lacZ*-containing plasmid, pCH110, identified those embryos which carried the transgene.

Breeding stocks were established from each transgenic mouse. The transgene was maintained heterozygously on a pigmented CBA background. Heterozygous transgenic embryos were obtained for experiments by mating transgenic males with wild-type CBA females. 0.5 day gestation (E0.5) was taken as noon on the date that female vaginal plug was noticed.

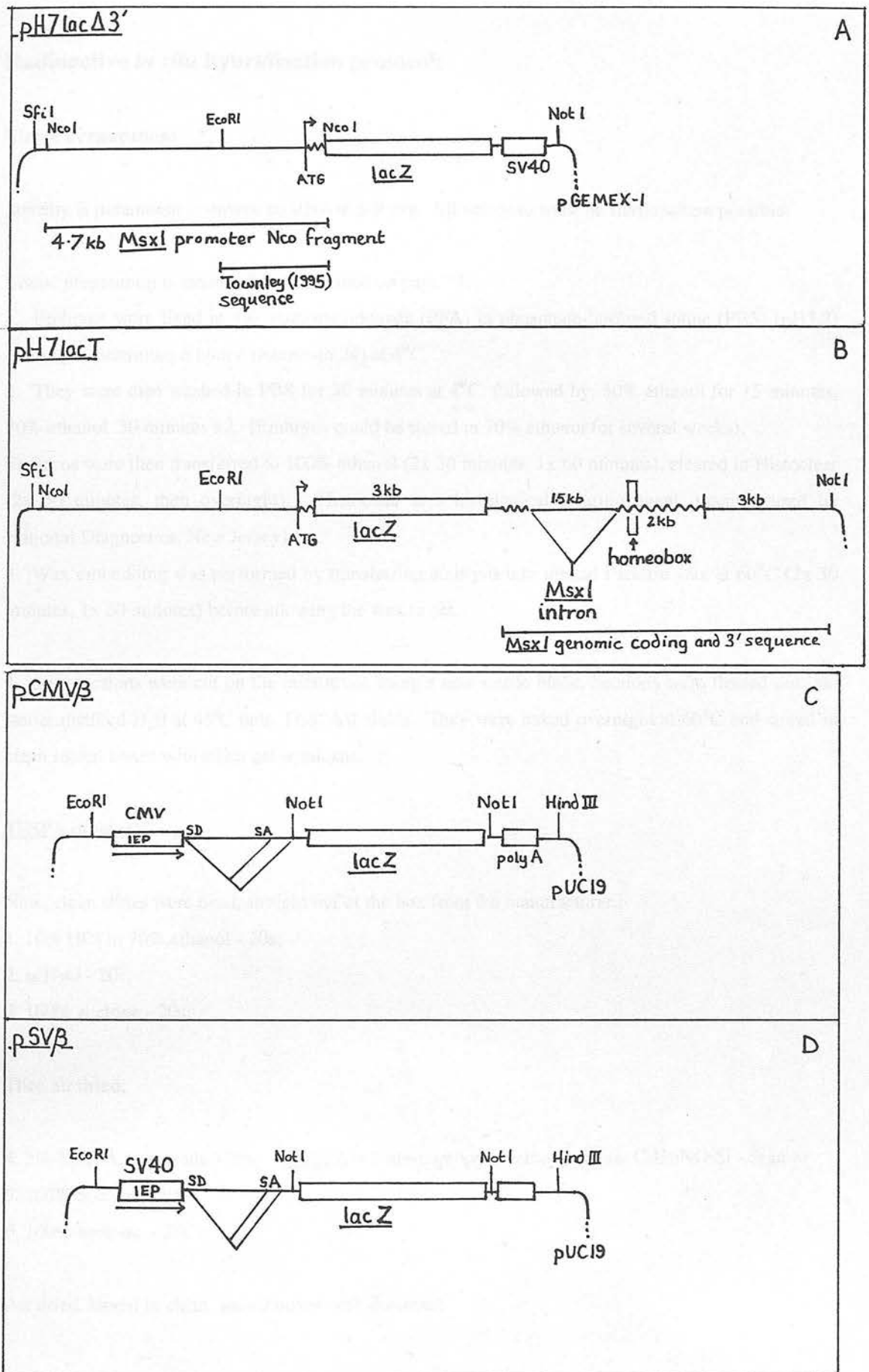


Figure 2.1: The plasmids pH7lacΔ3' (A), pH7lacT (B), pCMVβ (C) and pSVβ (D) inserts. Bacterial plasmid sequences not shown.

Radioactive *in situ* hybridisation protocol:

Tissue Preparation:

Sterility is paramount to ensure no RNase activity. All solutions must be sterile where possible.

Tissue preparation is essentially as described on page 93.

1. Embryos were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) (pH7.2) overnight (minimum 6 hours; maximum 24) at 4°C.

2. They were then washed in PBS for 30 minutes at 4°C, followed by, 30% ethanol for 15 minutes, 70% ethanol 30 minutes x2. (Embryos could be stored in 70% ethanol for several weeks).

Embryos were then transferred to 100% ethanol (2x 30 minutes, 1x 60 minutes), cleared in Histoclear (2x 30 minutes, then overnight). (Histoclear is a histological clearing agent, manufactured by National Diagnostics, New Jersey).

3. Wax embedding was performed by transferring embryos into melted Paraffin wax at 60°C (2x 30 minutes, 1x 60 minutes) before allowing the wax to set.

4. 6µm sections were cut on the microtome using a new sterile blade. Sections were floated out into sterile distilled H₂O at 45°C onto TESPA'd slides. They were baked overnight at 60°C and stored in clean sealed boxes with silica gel dessicant.

TESPA protocol:

New, clean slides were used, straight out of the box from the manufacturer.

1. 10% HCl in 70% ethanol - 20s;

2. sdH₂O - 20s;

3. 100% acetone - 20s;

Then air dried;

4. 2% TESPA in acetone - 20s; (TESPA = 3-aminopropyl-triethoxy silane, C₉H₂₃NO₃Si - Sigma)

5. 100% acetone - 20s

6. 100% acetone - 20s

Air dried, stored in clean, sealed boxes with dessicant.

TESPA slides were always prepared immediately before sectioning, although they can be stored for up to 4 weeks.

Probes for chicken and mouse *Msx1* and *Msx2*:

The DNA templates for mouse *Msx1* and *Msx2* - *pHox7.1* and *pHox8.1* - were as described by Monaghan *et al.* (1991). For *Msx2*, the *pHox8.1* plasmid is linearised with *Bam*HI and transcribes 350bp of *Msx2* 3'ORF and 3'UTR from the T7 promoter. The *pHox7.1*, *Msx1*, probe is linearised with *Bss*HII and transcribes the 3' 400bp *Bss*HII/*Eco*R1 fragment of *Msx1* cDNA (described by Hill *et al.*, 1989) from the T7 promoter. The two probes are absolutely specific for *Msx2* and *Msx1* respectively.

Probes for the chicken *Msx* genes - *Ghox7sp* and *G#p8.6* - were a gift from Sarah Wedden at the University of Edinburgh Department of Anatomy, and have been described previously. They too are specific for the 3' and untranslated regions of the genes. *Ghox7sp* contains the 800bp *Pst*I/*Eco*R1 *Ghox-7* cDNA fragment cloned into pBS SKII. It is linearised with *Bam*HI and transcribed with T7 polymerase. *G#p8.6* contains the 700bp *Pst*I *Ghox-8* cDNA fragment cloned into pBS SKII. It is linearised with *Bam*HI and transcribed with T7 polymerase.

Sense controls for all probes were transcribed using T3 polymerase. Sense controls were used during all the *in situ*s described in this thesis, and showed very low-level, uniform, background hybridisation.

Probe labelling reaction:

A ³⁵S labelled, ssRNA probe was transcribed from a dsDNA template by the following protocol:

In this order, add to a sterile Eppendorf:

3µl 5x transcription buffer (for T3 or T7 polymerase)

1µl 10mM rATP

1µl 10mM rCTP

1µl 10mM rGTP

1µl 1M DTT (Dithiothreitol)

6µl H₂O

12µl ³⁵S rUTP (>1mCi/100ml)

5µl template DNA (0.5-1mg/5ml)

(The DNA was double-stranded, linearised with the appropriate restriction enzyme and GENE-CLEANED twice according to the manufacturer's (BIO 101, Inc.) protocol.)

1.2µl RNase block
0.8µl T7 or T3 polymerase (Boehringer Mannheim), as appropriate.
Incubated at 37°C for 25 mins.

A further 0.8µl T7 or T3 polymerase was then added.
Incubated at 37°C for 25 minutes

Then add
2µl tRNA (10mg/ml)
1µl RNase-free DNase (Boehringer Mannheim)
Incubated at 37°C for 10 mins

Then added
2µl 100mM EDTA
167µl TE with 50mM DTT
(TE - 10mM Tris, 1mM EDTA, pH8)

The mixture was stuck on ice, transferred to a Microcon (Amicon) tube and spun in a bench centrifuge for 11 minutes.

Another 200µl of TE/DTT was added to the old tubes and then transferred to the Microcon tube, which was spun for a further 5 minutes. This gets as much probe as possible onto the Microcon filter.

The filter was then transferred to a blank Microcon tube (same way up) and 25µl TE/DTT was added. The tube was whirlmixed.

After cutting the top off the tube, the filter was turned upside-down and spun out for 2 minutes. The liquid spun out (containing probe) was transferred to a new screw-top tube.

The filter was turned over again and placed in a new blank Microcon tube. A further 25µl TE/DTT was added and vortexed. The filter was turned over again and spun out again for 2 minutes. The spun-out liquid was added to the previous aliquot in the screw-top tube.

The probe was placed on ice.

1µl aliquots of probe were taken for counting:

Scintillation counting:

1µl probe was added to 19µl TE/DTT and mixed.
10µl was added to each of two Whatmann GF/B filter

One filter was washed 3x in TCA (trichloroacetic acid solution - Sigma) and then once in ethanol. The TCA was allowed to stand on the filter for a few seconds to allow precipitation of the RNA before being drawn through by a vacuum.

Both filters were left to dry in air.

Unwashed and washed filters were counted separately in scintillation vials with 10µl Aquasol added, assuming 50% counting efficiency

A Packard Tri-Carb 1500 Liquid Scintillation Analyser was used, which is, literally and metaphorically, a black box to me.

$(\text{precipitate count})/(\text{total count}) \times 100 = \% \text{incorporation}$, which should be at least 10%

The probe was diluted appropriately with TE/DTT such that when Hybridisation mix was added in a 1:9 ratio, the final count would be 1.1×10^5 dpm/ml (dpm=disintegrations per minute).

Hybridisation mix (Hyb. Mix):

This was made fresh to the following final concentrations:

50% formamide

10% dextran sulphate

1x Denhardtts solution (see below)

20mM Tris pH8

0.3M NaCl

5mM EDTA

10mM sodium phosphate

0.5mg/ml tRNA

Then immediately before use, added

50mM DTT

50x Denhardtts Solution:

5g Ficoll

5g Polyvinyl pyrrolidone

5g Bovine serum albumen

Made up to 500ml with dH₂O, then filter-sterilised and stored at -20°C. This blocks non-specific attachment of probe to sections.

A 1ml batch of Hyb. mix is sufficient for over 20 slides.

Probe was added (1 part probe to 9 parts Hyb. Mix). The probe was heated to 80°C for two minutes, cooled on ice and added to Pre-hyb treated specimens.

Pre-hybridisation treatment of slides (Pre-hyb.):

Slides were taken through the following sterile solutions in batches of 20.

1. Xylene, 5 minutes x2
2. 100% ethanol, 2 minutes x2
3. 90%,70%,50%,30% ethanol, 2 minutes each
4. PBS 2 minutes with agitation
5. 4% PFA, pH7.2, 10 minutes

Slides were split into 2 racks of 10 to ensure no PFA remained caught between them.

6. PBS 2 minutes x2

Slides were drained and laid horizontal, then lined up back-to-back for Proteinase K treatment.

7. 20mg/ml Proteinase K in 50mM Tris, 5mM EDTA - 7.5 minutes

8. PBS, 1 minute

9. 4% PFA, 2 minutes

10. Distilled water, 10s

11. 0.1M TEA (triethanol amine) pH8 - 30s

12. 0.1M TEA + 625ml acetic anhydride - 5 minutes x2 with stirring

13. PBS - 2 minutes

14. 0.85% NaCl - 2 minutes

15. 30%, 50%, 70%, 90% ethanol - 1 minute each

- 16 100% ethanol - 3x5 minutes

Air dry in a dust-free box in two racks of ten slides.

Hybridisation of probe to specimens:

Probe/Hybridisation-mix mixture was heated to 80°C, then rapidly cooled on ice

Probe was then added to sections. 20µl covers 1/3 of the slide.

Coverslips, cleaned with ethanol, were put on the slides. Slides were placed horizontally in a sealed box, including a tissue soaked in 5mls of 50% formamide, 5x SSC. The box was sealed inside two plastic bags and submerged in a waterbath at 55°C for 16-18 hours.

(20xSSC: 0.3M Tris Na citrate, 3M NaCl, pH7.4)

Post-hybridisation washes:

1. 5xSSC, few seconds, 55°C
2. 5xSSC, 10mM DTT, 55°C, 15-30 minutes, until the coverslips fall off
3. **High stringency wash**, 65-68°C, 30 minutes

(**High stringency wash**: 50% formamide, 2xSSC, 0.1M DTT (add DTT just before use))

4. NTE, 5 minutes x2, 37°C

(NTE: 0.5M NaCl, 10mM Tris, 5mM EDTA, pH7.5)

5. NTE + 2µl/ml RNase A, 37°C, 30 minutes (In 30ml Coplin Jars)

6. NTE, 37°C, 30 minutes

7. High stringency wash, 65-68°C, 30 minutes

8. 2xSSC, room temp., 10 minutes x4

9. 0.1xSSC, room temp., 5 minutes x4

10. 30%, 50%, 70%, 90% ethanol, 1 minute each

11. 100% ethanol, 5 minutes x2

12. Air Dry.

The slides were dipped in Ilford K5 emulsion at 40°C

(7mls sdH₂O with 7ml emulsion, mixed and melted at 40°C. Slides dipped back to back in slide dipper, then separated and allowed to dry in a perspex rack for 3 hours. They were then transferred to a sealable box with dessicant, covered with foil and left at 0-4°C for 2-3 weeks before developing

Developing slides:

All solutions at room temperature.

Slides immersed in;

1. D19 developing solution - 4 minutes
2. dH₂O - 10s
3. 1:2 fixative (AMFIX) - 5 minutes
4. dH₂O - 10 minutes x2

and then counterstained in methyl green and mounted in **DPX**.

(**DPX** - xylene-based mounting medium, manufactured by BDH Laboratory Supplies)

Whole-mount *in situ* hybridisation protocol:

All the whole-mount digoxigenin *in situ*s presented in this thesis were performed by Lesley McGinnis and Liz Graham at the MRC Human Genetics Unit. This was performed essentially as described by Wilkinson and Nieto in *Methods in Enzymology* **225**, 361-373 (1993). The subsequent analysis of these *in situ*s was my own work.

The DIG DNA labelling kit (Boehringer Mannheim) was used to T7 transcribe the template and label with digoxigenin. After ethanol precipitation of the labelled probe and alkaline hydrolysis, hybridisation of probe to prepared tissue was performed as described in the above reference. The DIG detection kit (Boehringer) was used to stain hybridised transcripts.

Many of these limbs were prepared in the early days of whole-mount *in situ* and the technique has been improved subsequently. This, and the fact that the limbs were stored in fix for 12 months at 0-4°C before they were made available to me, raised the question of sensitivity and reliability of the data. While the whole-mounts are undoubtedly not as sensitive as radioactive *in situ*s, (expression of *Msx1* in the AER is at the limit of sensitivity for the whole-mounts, but is well above background in radioactive techniques), data taken from them agrees in all cases with the more limited data from radioactively probed tissue sections.

The benefits of the whole-mount technique, in the larger numbers of specimens it is possible to process, and the more complete picture of expression in space and time, far outweigh the disadvantages of sometimes having to strain ones eyes down the stereomicroscope.

Eukaryotic cell culture:

Basic cell culture:

Cells were cultured in 80ml tissue culture flasks in complete growth medium, usually Dulbecco's Modified Eagle Medium (DMEM) (Gibco-BRL) containing 10% heat-inactivated foetal calf serum (FCS).

Mouse primary limb mesenchyme cells were kept in CMRL1066 medium (Gibco-BRL) with 1% galactose. All cells were maintained at 37°C, 5% CO₂.

Cells were split when they reached confluence; all manipulations were performed in a laminar air flow cabinet.

1. Medium aspirated from cells.
2. Cells washed briefly in PBS at 37°C.
3. PBS removed and 6ml of Trypsin/Versene 1:10 solution (0.2% trypsin, 0.04% EDTA in Dulbecco 'A') was added for 5 minutes or until cells dislodged from the flask.
4. 4ml of complete growth medium added to dilute the trypsin and reduce the possibility of cell death.
5. Cell suspension transferred to a 10ml Sterilin conical tube and cells spun down at 2000rpm for 5 minutes in a refrigerated centrifuge at 4°C.
6. Supernatant aspirated away and cells gently resuspended in 10ml fresh medium using a pastette.
7. ~2ml cell suspension was then added to 25ml fresh complete medium in a flask and returned to the incubator.

Protocol was essentially the same for cells in tissue culture petri-dishes, although the volumes of the various reagents obviously had to be adjusted.

To minimise passages of the cells, a stock of each line stored in liquid nitrogen, from which fresh samples could be taken every month or so. This minimised the risk of a population of spontaneously differentiated or transformed cells occurring during the time-course of the experiments.

Freezing cells;

1. Cells in flask washed once with PBS.
2. PBS aspirated off and cells trypsinised as above.
3. Cells were then spun down, as above.
4. The pellet was taken up in ~3ml of 'freezing mix' - FCS with 10% DMSO (dimethylsulphoxide)

5. 1ml aliquots of cell suspension were pipetted into Nunc Cryotubes.

6. Cryotubes were frozen at -70°C overnight and transferred to liquid nitrogen next day, where they could be stored indefinitely.

Thawing stored cells;

1. The frozen cryotube was thawed quickly by plunging it into a beaker of water at 37°C .

2. Cells were added to 9ml medium in a Sterilin conical tube.

3. Then spun down as above.

4. The pellet of cells was resuspended in 5ml medium and added to 25ml complete growth medium in a flask, then placed in the 37°C , 5% CO_2 incubator.

Preparing cells for microinjection

Cells were injected as a monolayer on the culture dish. Cell lines such as 10T^{1/2} and COS cells therefore needed no special preparation. Primary limb mesenchyme cells needed plating out after isolation from the limb.

Plating out primary limb mesenchyme cells for microinjection:

The protocol was essentially the same for mouse and chick cells of the required stage. E10.5 mouse embryos were taken from the pregnant mother and kept on ice in PBS while the fore-limbs were removed with sharpened forceps. Chick embryos were taken from the egg and the fore-limbs removed into cold PBS.

1. Fore-limbs placed in a 1:10 solution of Trypsin/Versene (0.2% trypsin, 0.04% EDTA) at 4°C for one hour until the outer ectoderm sheath started to peel off.
2. All the ectoderm was then carefully removed and discarded using sharpened tungsten needles. These were prepared, as described by Dossel (1958), by repeated immersion of needle tips in molten sodium nitrite in a porcelain crucible. Needles were washed with distilled water before use.
3. Distal mesenchyme was cut, using tungsten needles into small pieces (100µm or smaller).
4. Mesenchyme pieces were then transferred (carrying as little trypsin solution as possible) into complete growth medium in 60mm tissue culture dishes. For chickens, this was DMEM + 10% FCS, for mice, CMRL1066 medium, 1% galactose and 10% FCS.
5. After a couple of hours, the pieces stuck down on the dish, and overnight started to grow out as a monolayer of cells around the central blob. These were suitable for microinjection. Cells would survive for several days and begin to differentiate, though the death rate of chicken cells was significant.

Preparation of DNA for microinjection:

Plasmid DNA was maintained in bug stocks at -70°C . Circular plasmid DNA was prepared by one of the following two methods, depending on the amount required and time available.

Plasmid preparation - isolation of plasmid DNA from cultures of *E.Coli*:

Maxipreps - for isolating up to 1g of plasmid DNA;

1. *E.Coli* maintaining the required plasmid were grown overnight in 500ml **Terrific broth** at 37°C with shaking so that stable phase of population growth was reached.

Terrific broth:

12g Bacto-trypton (Difco)

24g Yeast extract (Difco)

4g glycerol (Sigma)

made up to 900ml with dH_2O , autoclaved, then 100ml of sterile phosphate buffer ($0.1\text{M KH}_2\text{PO}_4$, $0.72\text{M K}_2\text{HPO}_4$) added.

2. Culture transferred to 200ml centrifuge tubs. Spun at 5000rpm, 5 minutes.
3. Supernatant removed and pellet taken up in 20ml **GTE** buffer containing 10mg/ml lysosyme.

GTE buffer; 25mM Tris
50mM EDTA
1% glucose

in distilled water, filter-sterilised. pH8

4. 40ml **alkaline solution** added and suspension stood on ice for 10 minutes. This treatment releases cell contents by disrupting the cell walls and causing bursting. Alkaline solution is a denaturer.

Alkaline solution: 1% sodium dodecyl sulphate (SDS)
0.2M NaOH
in distilled water.

5. 30ml of 3M potassium acetate, pH5.5, was then added and the suspension shaken. Left on ice for 30 minutes.

This precipitates chromosomal DNA

6. Suspension spun at 12000rpm for 30 minutes. Then supernatant was passed through 4 layers of muslin and transferred to a new centrifuge bottle. The pellet, containing chromosomal DNA, was discarded.

7. 45ml of isopropanol was then added to precipitate plasmid DNA. Suspension was shaken and left to stand at room temperature for 10-15 minutes.
8. Suspension was again spun for 30 minutes at 12000 rpm.
9. Supernatant discarded and the pellet was rinsed in diethyl ether. Pellet was then dried briefly in a freeze dryer.
10. Pellet was dissolved in 3.3ml TE (10mM Tris, 1mM EDTA, pH8), then 3.55g CsCl and 200ml ethidium bromide (10mg/ml) added.

The refractive index of the mixture was then measured and small amounts of TE or CsCl were added to bring it to between 9'30 and 9'45.

11. Balanced samples were spun at 80000rpm for 16 hours to produce pink DNA bands in the CsCl gradient (Pink because the ethidium bromide binds DNA, unwinding it and also causing it to become fluorescent).

12. DNA band was gently sucked into a syringe and transferred to an Eppendorf tube. An equal volume of butan-1-ol was added. Tube was vortexed and the liquid then settled into a pink upper alcoholic layer containing the ethidium bromide and a colourless aqueous lower layer containing the DNA.

13. The top pink layer was removed and discarded. Equal volume of butan-1-ol were added, shaken and discarded, as above, 2-3 times until no pink colour was discernible.

14. 2.5 volumes of 75% ethanol were added to precipitate the DNA. Suspension transferred to -70°C for 20 minutes, then sample was warmed again to 37°C to dissolve the CsCl.

15. Balanced samples were spun in bench microcentrifuges for 20-30 minutes at room temperature. Ethanol supernatant was discarded. The pellet was resuspended in 400µl TE.

16. 40µl of 3M sodium acetate, pH 5.5 and 880ul of 100% ethanol were then added to the suspension, which was then cooled to -70°C for 20 minutes.

17. Spun in bench centrifuge for 10 minutes. The supernatant was discarded and pellet washed in 200ml of 75% ethanol to remove the acetate.

18. Spun again for 2 minutes in the bench centrifuge, ethanol was removed and the pellet dried for a couple of minutes in the freeze-drier.

19. Pellet redissolved in filter-sterilised TE.

20. The suspension was usually RNAsed by adding 2µl of 10mg/ml RNase-A stock and incubating at 37°C for 15-30 minutes.

21. A 1µl sample of the DNA was run on a 1% agarose gel in **TBE** to check for concentration and quality.

5x **TBE**; 270g Tris

138g Boric acid

23.3g EDTA

made up to 5l with dH₂O

Minipreps - A quick method of isolating small quantities ($\leq 10\text{mg}$) of plasmid DNA from *E.Coli*.

1. A single colony of *E.Coli* containing the required plasmid was grown overnight at 37°C with shaking in 5ml Terrific broth containing $100\mu\text{g/ml}$ ampicillin (to maintain selection for the plasmid).
2. Next day, 1ml of sample was taken and placed in an Eppendorf. Spun on bench microcentrifuge for 5 minutes.
3. Supernatant was discarded. Pellet was resuspended in 100 μl of freshly-made **Solution 1**.

Solution 1; 50mM glucose

25mM Tris

10 mM EDTA

2mg/ml lysozyme

pH8

4. Left on ice for 10 minutes.
5. 200 μl of **Solution 2** was added, and the mixture left on ice for a further 10 minutes.

Solution 2; 0.2M NaOH

1% SDS

6. 150 μl of 3M sodium acetate, pH5.5 was added, then vortexed and spun on the bench microcentrifuge for 10 minutes.
7. The supernatant was transferred to a new tube, and the pellet, containing chromosomal DNA, discarded.
8. 4-5 drops of 5M NaOH were added to the supernatant so that the pH was greater than 8.
9. 450 μl of phenol were added. The mixture was vortexed, then spun in the microcentrifuge for 3 minutes.
10. The upper aqueous phase, containing plasmid DNA was transferred to a new tube. The lower phenolic phase containing protein was discarded.
11. 400 μl of a 1:1 mixture of phenol:chloroform was added and vortexed. The mixture was then spun down in the microcentrifuge for 3 minutes.
12. The aqueous upper phase was transferred to a new tube and 240 μl isopropanol added. The mixture was vortexed and spun in the microcentrifuge for 15 minutes to pellet precipitated DNA.
13. Isopropanol supernatant was removed and the pellet washed in 200 μl 70% ethanol. Then back to the microcentrifuge for 3 minutes.
14. The pellet was dried briefly in the freeze drier and resuspended in 50 μl of TE.
15. Quality and quantity of DNA could be determined by running 1 μl on an agarose minigel with $\lambda\text{HindIII}$ and ΦX markers. RNA could be eliminated by adding 1 μl of RNase A stock solution and incubating at 37°C for 20 minutes.

Purifying DNA for microinjection:

DNA for microinjection into the cell nucleus must obviously be free of any salts, proteins and extraneous nucleic acids which could affect normal nuclear function.

1. DNA was prepared by the maxiprep or miniprep methods described above and treated with RNase A.

2. Phenol extraction;

To the 400µl of DNA in TE, 400µl of phenol (saturated with TE, pH8) was added. The mixture was vortexed then spun down for 2 minutes in an Eppendorf microcentrifuge. The soluble proteins coagulate in the phenol. When the mixture was spun it separated into two layers, a lower layer of phenol and an upper aqueous phase containing the DNA. The coagulated protein was visible at the interface.

3. The upper aqueous layer was transferred to a new tube, taking care not to disturb the crud at the interface. The lower phase was discarded.

4. The phenol extraction was repeated 2-3 times until no more protein appeared at the interface.

5. Ether was used to remove residual phenol in the aqueous phase;

400µl diethyl ether was added to the DNA solution. Vortexed and spun in the bench centrifuge for 2 minutes.

The liquid separated into two phases - an upper layer containing ether and phenol, and a lower aqueous layer with the DNA. The upper layer was removed and discarded.

This step was repeated twice. On the second repeat, residual ether was evaporated at 37°C for a few minutes.

6. Ethanol precipitation;

To the 400µl DNA solution in the Eppendorf, 40µl of 3M sodium acetate was added, and 880µl 100% ethanol. The mixture was frozen at -70°C for 10-20 minutes then spun in a microcentrifuge for 10 minutes. The ethanol was poured off.

Pellet was washed in 200µl 70% ethanol and spun for two minutes. The ethanol was removed and the pellet dried for a few minutes in a vacuum chamber.

7. The DNA was resuspended in 400µl of sterile 'injection' TE (10mM Tris, 1mM EDTA, pH7.05).

From this point, great care had to be taken to ensure the DNA was kept sterile.

8. Flushing out residual salts by dialysis:

This was performed normally, but I noticed no difference in terms of cell survival when undialysed DNA was used. The loss of DNA sterility and yield probably outweighed the benefits of salt-removal.

Dialysis tubing (stored in ethanol at 4°C) was washed several times in sterile TE, clipped at one end, the DNA introduced, then the other end was sealed. The tube was then placed in 2dm³ of sterile injection TE, which was changed after 1 hour and placed at 4°C overnight, with stirring.

9. The DNA was placed in a sterile Eppendorf tube which had been rinsed several times in filter-sterilised distilled water. It was divided into 10µl aliquots and stored frozen at -20°C and defrosted as little as possible to prevent activation of any residual DNase activity in the solution.

A 1µl sample of the DNA was taken and run on a 1% agarose minigel (containing 1µl 10mg/ml ethidium bromide) with known amounts of size markers λ /HindIII and Φ X. DNA concentration could be estimated from the intensity of the resultant bands. Required injection concentration was found not to be critical, but 50-100ng/ml was usually used.

10. If DNA picked up particles of dust during use, it could be filtered through a 0.22µm cellulose acetate filter in a Spin-X tube (Costar) in a bench centrifuge for 3 minutes. This removes any bacteria or solids which found their way in and which could block the injection needle or infect the cell.

Microinjection protocol:

Cell nuclei were microinjected using the Zeiss Axiovert Automated Injection System as a monolayer on 60mm tissue culture dishes in complete growth medium.

95-100% confluent cells were used. Over-confluent cells were not suitable for injection, as it was necessary to get all the cell nuclei at the same vertical level above the dish surface, since for a field of cells which would be injected at one time (as seen on the video screen), the injection needle could only be set to one vertical height. Hence, what the automated system gains in speed, it loses in accuracy. Other foibles of the system were that if a cell nucleus was missed, it was impossible to go back to it. Also, it was not possible to set a fixed volume of DNA which could be injected into the cell, only a pressure with which the DNA solution was forced in, and the length of time which the needle remained in the cell.

The injection needles used were sterile, pre-packed Femtotips (Eppendorf) with a theoretical tip diameter of $0.5\mu\text{m}$, although this varied. If these were not available, home-made, ethanol-sterilised pulled glass capillaries were used - these seemed to work just as well.

Cells were injected in 60mm tissue culture dishes at room temperature in complete growth medium under a thin layer of light paraffin oil which prevented airborne contamination reaching the cells during injection and prevented loss of CO_2 from the medium. Cells were removed from the incubator for as little time as possible, and were never allowed to cool down for more than 2 hours.

A grid of 2mm squares was scored on the underside of the dish with a scalpel blade mark the areas which had been injected (Fig. 2.2 (i)).

In each 2mm square, a set of 500 cells in the centre of the square was the target for injection (Fig. 2.2 (ii)).

As stated above, it was not possible to set a fixed volume of DNA to be injected (20pl is normally recommended). Injection pressure was therefore set so that during an injection time of 0.2s, a successfully hit nucleus would swell by $\sim 1/5$ in diameter. It was possible to see the refractive effect of the liquid entering the nucleus under the phase-contrast optics.

It was frequently necessary to reset the vertical height of the injection capillary, due to the bottom of the dish never being completely flat on a μm scale.

After injection, cells were allowed to recover in the incubator for at least 4 hours before further manipulation.

Preparing cultured injected cells for grafting:

Cells must be fully or very nearly confluent to survive this process in an intact sheet. There must be enough contact between cells to keep them together in the absence of a plastic substrate.

1. The cells were removed from the incubator four hours after injection. Using a sterile scalpel blade, a 1.5-2mm square was cut in the sheet of cells which coincided with the square on the underside of the dish and which would therefore contain a subset of injected cells (Fig. 2.2 (iii)).
2. This square of cells was then gently scraped from the dish in an intact sheet using a sterile 'rubber policeman'(!) - a short length of thin rubber tubing cut an oblique angle at the tip and anchored in a glass pipette at the other (Fig. 2.2 (iv)).
3. The sheet of cells was then gently sucked up with a drop of medium into a glass pastette whose end had been blunted by briefly passing it through a gas flame. Cells were deposited in pre-warmed complete growth medium in a 55mm Sterilin non-tissue culture petri dish and returned to the 37°C, 5% CO₂ incubator overnight. In these dishes, the cells could not stick to the plastic, and rolled up into a ball (~200µm) which was suitable for grafting.
4. The balls of cells could now be treated exactly like limb mesenchyme for grafting into the limb as described on page 89.

Preparation of limb mesenchyme for grafting:

1. Embryos were taken from pregnant female mice, and dissected out into cold PBS in a petri dish. The limbs were dissected off and placed in cold 0.2% trypsin (in 1:10 trypsin/versene solution). This was allowed to stand at room temperature for 20 minutes.
2. Using sterile tungsten needles, prepared as described earlier, the ectoderm was teased off, taking careful note of the orientation of the mesenchyme. 200µm fragments of mesenchyme were dissected from the required area of the limb, and removed, using a fine-tipped pastette, into cold CMRL1066 medium with 10%FCS, where they could be stored for up to 4 hours whilst grafting.

Embryos obtained from eggs which were obtained from the Adult Primary Research Centre at Bangor. These include the 2nd and 3rd stages of development, which are used for the purpose of the present study.

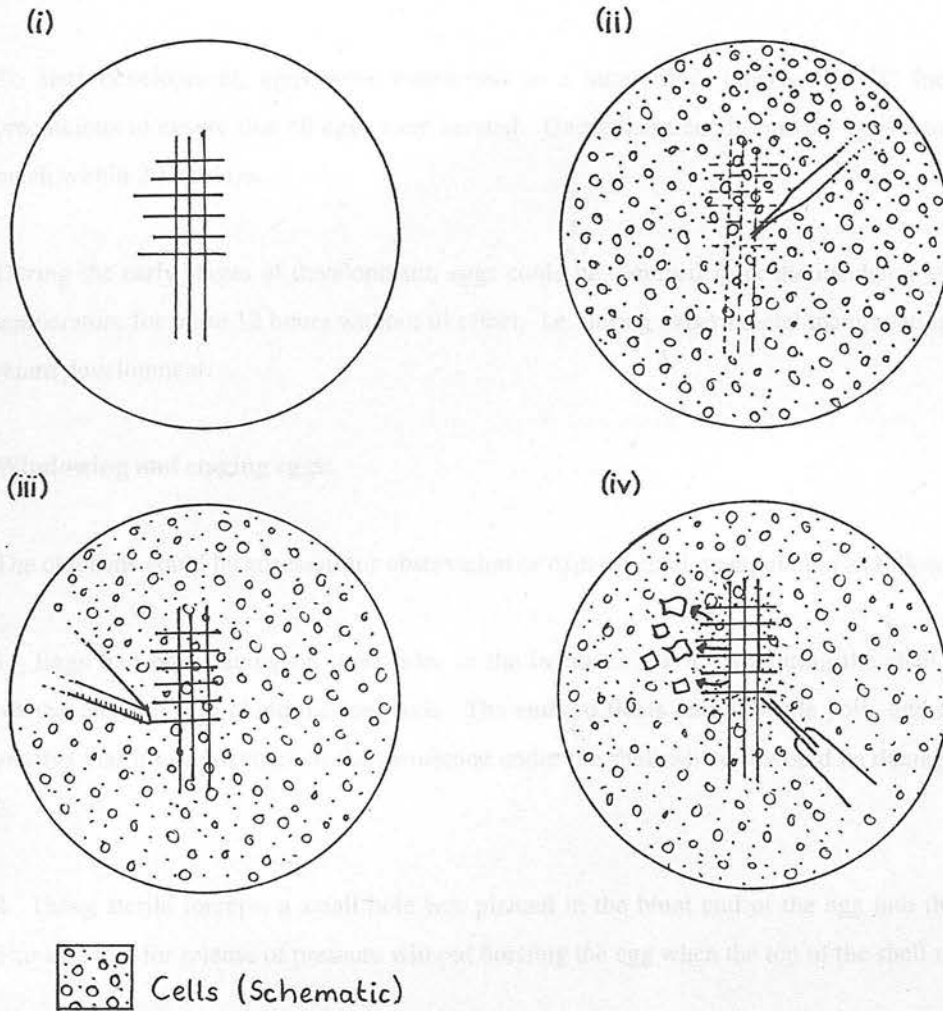


Figure 2.2: Microinjection protocol.

- (i) 2mm x 2mm squares scored on bottom of dish.
- (ii) Cells grown to confluence and those within the squares injected.
- (iii) Squares cut, in sheet of cells, which overlay those scored on underside of dish.
- (iv) 'Rubber policeman' used to scrape cells from dish, ready for transfer to a non-tissue-culture petri-dish, overnight.

Egg husbandry:

Fertilised chicken eggs (Ross White) were obtained from the AFRC Poultry Research Centre at Roslin. Upon receipt, they could be stored for up to a week at 12-15°C without development or death.

To start development, eggs were transferred to a humidified, ungasged, 38°C incubator, taking precautions to ensure that all eggs were aerated. Under these conditions the eggs would, if allowed, hatch within 20-21 days.

During the early stages of development, eggs could be removed from the incubator and left at room temperature for up to 12 hours without ill effect, i.e. during experimental manipulation or in order to retard development.

Windowing and staging eggs:

The chickens could be accessed for observation or experimental manipulation as follows;

1. Eggs had been laying on their sides in the incubator. Prior to cutting the shell, all eggs were rotated one half turn along the long axis. The embryo floats on top of the yolk, and this manoeuvre ensures that it has not stuck to the membrane under the shell where it would be damaged during Step 2.
2. Using sterile forceps, a small hole was pierced in the blunt end of the egg into the air chamber. This allowed for release of pressure without bursting the egg when the top of the shell was pierced.
3. An approximate 3x2cm piece of sellotape was stuck onto the top of the egg above where the embryo would be floating. Using sterile forceps, a small hole was punched through the shell through the sellotape. With this as an entry point, a 2x1.5cm oval could be cut in the top of the shell using fine sterile scissors.
4. With the embryo now visible, the eggs could be staged according to Hamburger and Hamilton (1951)
5. The egg could then be resealed with sellotape and replaced in the 38°C incubator where development continued as normal.

Grafting ectopic tissue into chick wing buds:

1. The egg was windowed and staged as described above.
2. Using sterile forceps the chorioallantoic and amniotic membranes on top of the embryo were broken and stripped away to give access to the chick.
3. Grafting sites were cut using tungsten needles.
4. Grafts were performed into sites as described below, i.e. either;

(i) **Distally** into the Progress Zone of a stage 20 wing bud. To do this, a slit of approximately the size shown was cut right through the limb just under the AER.

A ball of cells to be grafted, about 200µm in diameter was then delivered *via* a fine pulled glass pipette in a drop of medium such that it rested on the limb. The tungsten needles were then used to manipulate the AER and ball of cells such that the graft material was held in place at the distal tip of the wing bud by tension in the AER (Fig. 2.3a).

If the graft was positioned correctly, it would stay at the distal tip of the wing during the time course of the experiment.

(ii) **Proximally** into the stage 23/24 wing bud. A slit would be cut into the limb, about 1/3 of the way along, as shown, trying not to cut the main artery running into the limb and leaving a flap of ectoderm which could be used to cover the graft tissue after insertion. A 200µm ball of graft tissue was then introduced with a drop of medium as before, and embedded in the dorsal half of the limb just under the ectoderm such that it was held in place by compression of the surrounding mesenchyme (Fig 2.3b).

(iii) Grafts to the **anterior** margin of the stage 23 bud were performed by using the tungsten needles to cut a slit through the anterior mesenchyme so that the anterior epithelium lifted away (like the AER in (i)). A 200µm ball of cells could then be inserted into the gap and held in place by pressure of the surrounding mesenchyme (Fig. 2.3c).

(iv) Grafts to the **flank** mesoderm, anterior to the limb, were performed by cutting a slit in the body wall, between the somites and the aorta. The cells could be placed in the resulting hole, held in place by the surrounding tissue (Fig. 2.3d).

5. The egg was then carefully resealed using sellotape and replaced in the incubator. Experimental manipulation, including rupture of the extra-embryonic membranes, did not adversely affect the further development of the egg, providing bleeding and other damage to the embryo had been kept to a minimum.

When the time came to remove the embryo from the egg for analysis of gene expression, it could be lifted out using blunt sterile forceps.

Other unused embryos up to 10 days of age were killed by freezing .

Grafts into mouse tissue in culture:

Grafts into mouse tissue in culture were performed in cold CMRL1066 medium with 10% FCS in 35mm tissue culture dishes. The mouse head or limb would be held gently with flame-sterilised, cooled, forceps whilst the graft site was cut with a tungsten needle. Grafting tissue was introduced with a fine-tipped pastette and manipulated with the needle until it held in place.

Mandibular grafts: The graft site was cut by approaching the mandibular process from the front and cutting a ventral-lateral slit as if making a transverse section of the head. The graft tissue was held in place by internal pressure of the displaced mandibular mesenchyme after grafting (Fig. 2.3e).

Maxillary grafts: Graft site was prepared as described for chickens by Brown *et al.* (1993). Approaching the head on its side, a slit was made underneath the maxillary epithelium, such that it could be pulled forward to admit the graft tissue. The pressure of the stretched epithelium held the graft in place subsequently (Fig. 2.3f).

The grafted heads and limbs were culture subsequently in CMRL1066 medium with 10% FCS, in a humidified chamber at 37°C and 5% CO₂. Importantly, the graft sites healed well and in most cases the epithelium reformed over the graft.

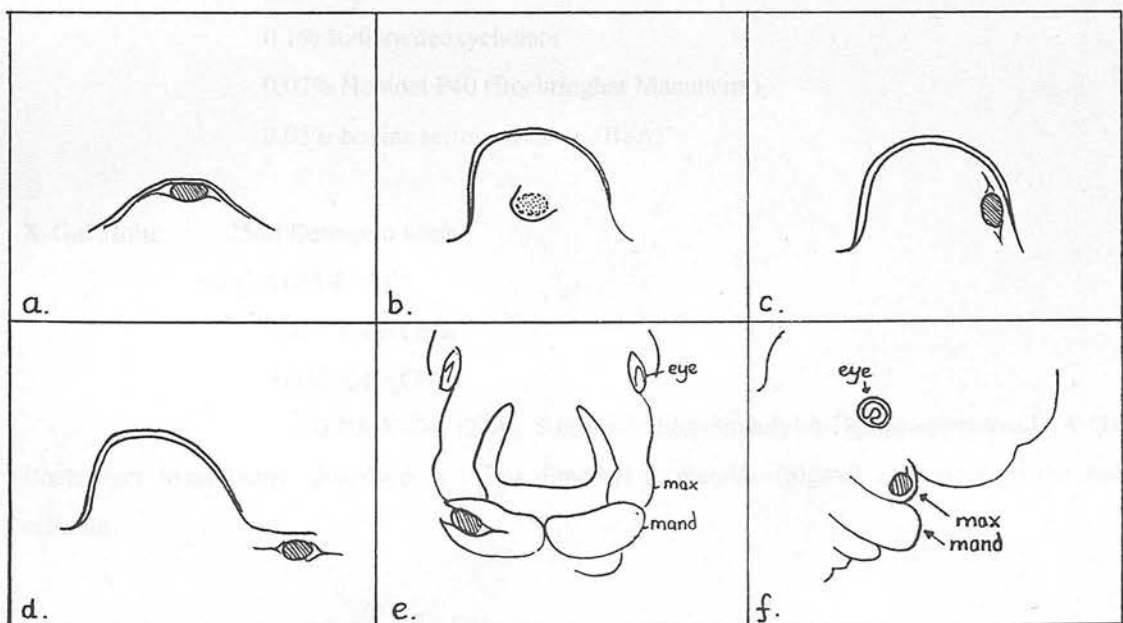


Figure 2.3: Grafts sites as described in text: a. distal; b. proximal; c. anterior; d. flank; e. mouse mandible; f. mouse maxilla.

X-Gal staining of chicken and mouse embryos:

1. Extra-embryonic tissue was removed from embryos and they were rinsed briefly with PBS.
2. Embryos were placed in excess **fix** solution for 1 hour at 4°C. (1ml fix per embryo).
3. Fix was pipetted off and embryos washed three times with an excess of **detergent wash** for twenty minutes each wash, at room temperature.
4. After last wash solution was removed, an excess of **X-Gal stain** was added and transferred to 37°C for 2-4 hours.

Any genuine staining was visible after two hours. The reaction conditions favour the activity of bacterial *lacZ* over that of the endogenous eukaryotic gene. Overnight incubation visualised endogenous β -Gal staining in the metanephros, heart and AER.

5. After staining, embryos were washed in PBS briefly then taken up through an ethanol series, 30%, 50%, 2x 70% for 20 minutes each and could be stored indefinitely at 4°C.

Fix Solution: 77ml 0.1M phosphate buffer

(Made by adding 126ml 0.1M $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ to 400ml 0.1M Na_2HPO_4)

2% formaldehyde (20ml Millory's 10% neutral buffered formalin - 10% formaldehyde in $\text{NaOH}/\text{NaH}_2\text{PO}_4$ buffer)

0.2% gluteraldehyde

2mM MgCl_2

5mM EGTA pH8

Detergent Wash: 468 ml 0.1M phosphate buffer

2mM MgCl_2

0.1% sodium deoxycholate

0.02% Nonidet P40 (Boehringer Mannheim)

0.05% bovine serum albumen (BSA)

X-Gal stain: 25ml Detergent wash

with 0.085% NaCl

5mM $\text{K}_3\text{Fe}(\text{CN})_6$

5mM $\text{K}_4\text{Fe}(\text{CN})_6$

0.1% X-Gal (25mg 5-brom-4-chlor-3-indolyl-b-Dgalactopyranosid, (X-Gal, Boehringer Mannheim) dissolved in 0.5ml dimethyl formamide (Sigma) and added to the stain solution.

The protocol for staining a cell monolayer was essentially the same. Fixation time was reduced to 15 minutes, followed by three washes of 10 minutes each in detergent wash. Staining still took 2-4 hours.

Quick X-Gal for identifying transgenic tissue:

Transgenic mice were maintained on a heterozygous background, and mated with wild-type females, so half the embryos of any litter produced were wild type. To identify transgenic embryos within an hour of collection, for the experiments described in Chapter 5, the following protocol was used.

1. Embryos dissected out into cold PBS. A part of each embryo which would not be required for grafting (usually the head or the hind-limb) was placed in a few drops of X-Gal fix (as above) in numbered wells of a 96-well tissue culture plate (Costar). The rest of the embryo was stored at 0-4°C in PBS in a 24 well plate.
2. After 10 minutes in fix on ice, with one change of solution, the piece of embryo was transferred to different numbered wells containing a few drops of Detergent Wash.
3. With two changes of solution, the specimens were washed for 10 minutes at room temperature before removing the wash and adding a few drops of pre-warmed stain at 37°C.
4. After 15 minutes at 37°C, blue staining in the AER or mandibular mesenchyme was easily visible. This allowed identification of transgenic embryos with 100% accuracy. They could be retrieved from the 24-well plates in PBS and prepared for grafting exactly as described on page 86.

Tissue preparation for light microscope examination

As will be seen from some of the figures, the following method was not ideal for tissue preparation. There was a problem with sectioning X-Gal stained embryos, when the fix did not adequately preserve morphology. I solved this partly by refixing embryos in 4% PFA after staining, but even this was not ideal. Alterations to the fixing and dehydration procedures recently performed by Liz and Allyson have improved the technique.

Wax-embedding of embryos:

1. Fixed embryos were washed in PBS for 30minutes.
2. PBS was removed and embryos washed in a 0.85% solution of NaCl in sdH₂O for 30 minutes.
3. 2x washes in 1:1 0.85% NaCl:ethanol, 15 minutes each.
4. Washed in 70% ethanol, 15 minutes. Then 2nd wash in 70% ethanol, 15 minutes-overnight.
5. Washed in 85% ethanol, 30 minutes.
6. Washed in 95% ethanol, 30-45 minutes.
7. 3 washes in 100% ethanol, 2x 30minutes and 1x 60 minutes.
8. Transferred to HistoClear. 2x30minutes.
9. Soaked overnight in fresh HistoClear.
10. Embryos were then embedded in paraffin (56°C wax). Each embryo was immersed in two changes of molten wax for 30 minutes each, then transferred to a final watchglass of molten wax for 60 minutes before the wax was allowed to cool, ensuring that the orientation of the embryo in the setting wax was known and that the embryo was not too close to the surface of the wax block. Embryos could be stored for prolonged periods in the wax blocks.

Sectioning and mounting wax blocks for light microscope examination:

1. 7µm sections were cut from the wax blocks on a hand-microtome.
2. 3-4 sections at a time were floated out onto distilled water at 45°C and allowed to stick to alcohol-cleaned, TESPA'd glass slides.
3. Slides baked at 60°C overnight to stick sections to slide.

Eosin Counterstaining

1. Slides de-waxed in xylene, 5 minutes x2
2. Rehydrated in ethanol series: 100%, 5 minutes x2; 90%, 5 minutes; 70%, 5 minutes; 50%, 5 minutes
3. Staining - 5 minutes in 1% eosin ('Eosin, Yellowish' - TAAB Laboratory Equipment Ltd.) in 50% ethanol, 5 minutes
4. Slides rinsed briefly in 50%, then 70% ethanol. Transferred to 100% ethanol (1 minute x2)
5. Slides washed 5 minutes x2 in xylene before mounting in DPX (Xylene-based mounting medium - BDH Laboratory Supplies).

Chemical Transfection Protocols:

Transfectam:

Transfectam is a cationic lipopolyamine, manufactured by Promega (Promega Technical Bulletin 116)

Plating cells for Transfection;

Cells were plated out into 60mm tissue culture dishes in serum-free medium such that on the day of transfection they were at the required confluence. 70% is recommended. Before transfection, the medium was removed and replaced with 500µl fresh serum-free medium.

Preparing Transfectam Reagent Stock;

1µg Transfectam was dissolved in 40µl 96% ethanol. After 5 minutes, 360µl of sterile distilled water was added and the solution was vortexed. Store at 4°C.

Preparing Transfection Solution;

Transfecting plasmid (1-5mg) was added to 500µl serum-free medium and vortexed. (Solution A)

For each mg of plasmid used, 1.5 to 5µl of Transfectam stock solution was added to 500µl serum-free medium and vortexed. (Solution B).

Solutions A and B were immediately mixed and added to the cells.

Cells were returned to the incubator overnight, and the medium replaced next day.

24 hours were allowed to elapse before checking transfection efficiency.

Calcium Phosphate Transfection;

The protocol used was similar to that described in 'Gene Transfer and Expression' (Kriegler).

1. Cells were plated out to the required confluence in 5ml fresh medium on 100mm tissue culture dishes.

2. Transfecting plasmid (5-20µg) in sterile **TE** pH7.05 was added to 0.45ml sterile water. 0.5ml 2x **HBS** was added. 50µl of 2.5M filter-sterilised calcium chloride was added and the mixture vortexed.

The mixture was allowed to stand for 20 minutes to obtain a milky precipitate.

TE - Tris-EDTA, pH7.05

0.01M Tris

0.001M EDTA

Filter-sterilised

2x HBS - HEPES buffered saline:

KCl 3.7g

D(+)glucose 10.0g

Na₂HPO₄ 1.0g

Made up to 50ml with sdH₂O

1ml of this salt solution was added to 1.0g HEPES (N-2-hydroxyethylpiperazine-N'2-ethane sulphonic acid) and 1.0g NaCl

Made up to 85ml with sd H₂O

1M NaOH was added to bring to exactly pH7.1

Solution made up to 100ml with sdH₂O

3. 1ml of the transfection mixture was added directly to the cells in culture, in medium.
4. Cells incubated at 37°C for 16 hours.
5. Medium with transfection cocktail was removed and replaced with fresh complete growth medium.
6. Cells were incubated for 24 hours before checking for transfection efficiency.

DEAE Dextran (Sigma) Transfection protocol:

1. Cells were plated out in 60mm tissue-culture dishes the day before transfection in medium such that they would be at the required confluence the next day.
2. 0.5-10mg of transfecting DNA was resuspended in 75µl TE pH8. 150µl TBS was added and 150-450µl **DEAE Dextran stock** solution.

DEAE Dextran stock; A 1mg/ml solution in TBS. Filter sterilised and stored at 4°C.

TBS- Tris-buffered saline;

10 ml of **solution 'A'** (see below) was added to 1ml **solution 'B'** and made up to 100ml with sterile water.

Solution 'A';

NaCl 8.0g

KCl 0.38g

Na₂HPO₄ 0.20g

Tris 3.00g

Made up to 100ml with sterile water, pH'd to 7.5 and filter-sterilised.

Solution 'B';

CaCl₂.2H₂O 1.5g

MgCl₂.6H₂O 1.0g

Made up to 100ml with distilled water. Filter-sterilised

3. All reagents were brought to room temperature
4. Growth medium was removed and cells washed once with 9ml PBS followed by one wash with 9ml TBS.
5. TBS removed and the DNA/DEAE Dextran/TBS mixture was added to the cells. Dishes of cells were then rocked at room temperature for one hour to ensure they did not dry out.
6. After 1 hour, the transfection mixture was removed. Cells were washed once with 9ml TBS and then once with 9ml PBS.
7. PBS was removed and 6ml complete growth medium, containing 100µM **chloroquine** was added to the cells. Returned to 37°C, 5%CO₂ for 4 hours.

Chloroquine (Sigma) stock solution; 100mM in sdH₂O. Filter-sterilised and stored at -20°C.

8. Chloroquine/media solution removed and replaced with chloroquine-free complete growth media.
9. Cells were incubated for a further 24 hours before assaying for gene expression.

Preparation of stably transformed cell lines:

This protocol was used to select for cells which have stably integrated the neomycin resistance gene, *neo^r*.

1. Cells were transfected by the calcium phosphate transfection method, as detailed above, using 1µg of the *neo^r*-containing plasmid, pMCneo and 10µg of the plasmid containing the gene under study. the plasmids can, but need not, be linearised.

2. 16 hours after transfection (this allows for DNA uptake and integration), growth media was removed and replaced with complete growth media containing 800µg/ml G418 sulphate (Geneticin sulphate, Paludin *et al.*, 1989).

(G418 sulphate stock solution - 10mg/ml in sdH₂O, filter-sterilised. Stored at -20°C)

3. Cells were maintained under selection by replacing the selection medium every 2-3 days. Otherwise, cells were cared for as normal.

Preparation of beads for implantation into embryos:

I 'borrowed' rhBMP4 from Richard Buckland, who obtained it from Vicki Rosen at the Genetics Institute.

A few microlitres of heperan acrylic beads (Sigma) in buffer were added to 100µl sterile PBS in a 55mm sterile petri dish.

Using sterile fine forceps, 200-250µm diameter beads were selected and transferred into 5µl drops of either 100ng/ml BMP4, 10ng/ml BMP4 (diluted in sterile PBS) or 0ng/ml BMP4 (sterile PBS) in separate 55mm petri dishes.

(The BMP drops were in the centre of the dish, which was kept moist with 10ml drops of PBS round the circumference)

10 beads were placed in each 5µl drop. They were left to soak for 1-2 hours at room temperature.

For implantation, beads were taken directly from these drops using fine forceps and placed into a site, cut in the limb with tungsten needles.

Isolation of pure linear DNA sequences, from circular plasmids, for mouse oocyte pronuclear microinjection:

1. About 20µg of the closed circular plasmid, isolated from the bugs by maxipreps (see earlier) was used.

2. Appropriate restriction enzymes were used to release the required insert. Digests were performed in a total volume 200µl, using incubation temperatures and buffers as per manufacturers instructions.

If, for a double digest, different buffer conditions were needed for the two enzymes, a digest would first be performed using one enzyme and its correct buffer, then the cut DNA resuspended in a small volume of TE using Microcon tubes (Amicon). The second digest would then be performed under conditions optimal for the second enzyme.

Microcon tubes pass the DNA through a membrane which retains all molecules greater than 30kD. The DNA solution to be de-salted is made up to 500µl with TE and spun in the Microcon tube for 10 minutes. Liquid which has passed through the membrane is discarded and the upper reservoir is refilled with TE. The tube is spun for a further 10 minutes and the liquid passing through is discarded. This step may be repeated if thought necessary. The upper tube with the filter is then turned round into a clean Eppendorf and spun again for 3 minutes. The DNA is released into the new tube in a small (<40µl) amount of TE.

3. To check that digestion had gone to completion, 5µl of the digestion mix was run on a 1% agarose gel in TBE, with uncut plasmid in another lane and size markers in others.

Gel photographed under UV light. There should be a clean band at a size corresponding to the required insert, other bands corresponding to other bits of the cut plasmid, and no uncut plasmid bands.

4. Providing digestion was satisfactory, the remaining digestion mix was purified using Microcon tubes, and resuspended in 60µl TE.

5. Isolation of the required insert from an agarose gel;

Agarose gels were prepared by heating mixed agarose and TBE in a microwave oven until the agarose dissolved, pouring the mixture into the gel tray while still hot, adding ethidium bromide (5-10µg per 20ml gel) and allowing to set.

A 0.8% low-melting-point (LMP) agarose minigel was poured with three middle lanes of the comb taped together to give one wide lane. 1µg ethidium bromide was added to the gel.

The 60µl of digested DNA in TE was mixed with 5ml methyl orange loading buffer and run in the wide central lane. Size markers were in the narrower outer lanes.

The gel was run at 50V in TBE buffer to stop it overheating.

The gel was allowed to run until the required band was well separated from its neighbours, then, with the DNA visualised under low intensity UV only (to prevent DNA damage) this band was quickly cut from the gel with a sterile blade and transferred to an eppendorf tube of known mass.

The mass of the gel/DNA was then measured.

The agarose was then digested using the *Pseudomonas atlantica* agarase protein (Boehringer Mannheim) according to manufacturers protocol.

0.04 volumes of 25x agarase buffer was added to the gel slice and incubated at 65°C for 15 minutes to melt the agarose.

The mixture was cooled to 45°C and 2 units of agarase were added per 100mg of agarose. Mixed well.

Incubated for 1 hour at 45°C to ensure that all agarose was digested.

6. Isolation of the DNA from the digested agarose using Elutip-d columns;

Elutip-d (Schleider and Schuell) columns were designed to isolate DNA from agarose digestions and remove impurities that often creep in during this treatment. The manufacturers protocol was used.

DNA was made up to 9ml with Low Salt solution.

Low Salt solution; 0.2M NaCl

20mM Tris HCl pH7.4

1mM EDTA

2ml High Salt solution was driven through the column to wash the matrix.

High Salt solution; 1M NaCl

20mM Tris HCl pH7.4

1mM EDTA

5ml of low-salt solution was then forced through the column to remove the high salt solution.

The DNA/salt solution was then slowly forced through the filter/column set up - DNA bound to the matrix in the column.

The DNA could be washed out from the column into an Eppendorf tube by passing 0.4ml of high salt solution through.

This protocol yields 400µl of pure insert in high salt solution.

7. The insert was resuspended in 50µl of TE by taking it through Microcon tubes as described earlier. Concentration of insert was estimated by running a 1µl sample against fragments of known size and concentration on a 1% agarose gel.

CHAPTER THREE

The expression and possible roles of mouse *regiment homeobox* genes, *Max1* and *Max2*, during mouse and chicken limb development: their potential for functional redundancy

RESULTS

Introduction

The generation by homozygous recombination of mice which were homozygous for a mutation in *Max1* was not possible because these mice showed no detectable limb phenotype (Santata and Meek, 1997). One possible explanation for this observation is functional redundancy between *Max1* and *Max2*, which is also expressed in the anterior and posterior of the developing limb bud (Carlier *et al.*, 1997a). To evaluate whether this explanation is plausible, one would need to know whether *Max2* is expressed in the cells which express *Max1* at each successive stage of limb development. To date however, there has been no comprehensive, detailed description of the gene expression in the mouse limb bud which to make this evaluation.

A knowledge of the expression patterns of the *Max* genes is fundamental to an understanding of their roles in the development of the embryo. Previous analysis of the expression of the genes has been limited by the low resolution of in situ methods, which restricts the number of embryos which can be processed and makes a three-dimensional reconstruction of expression patterns difficult. A series of whole-mount in situ hybridisation experiments in mouse limb buds was performed with Lisa Stedum and Emily McGuire between 1997 and 1998 using after printing on nylon and slides as probes.

I analysed the expression of *Max1* and *Max2* in the mouse limb to investigate the degree to which the expression patterns of *Max1* and *Max2* overlap. Expression of the chicken homologues (Suzuki *et al.*, 1991; Yokouchi *et al.*, 1991) was also analysed for two reasons. Firstly, although much information has been published previously about the expression of the genes in the chicken limb, there has been no comprehensive stage-specific analysis, and this has led to some confusion and controversy in the literature. Secondly, the history of classical embryological experiments on the chicken limb has led to operational definitions of regions such as the Zone of Polarising Activity (ZPA) (MacCabe *et al.*, 1972; Harig and MacCabe, 1976; Vogels, 1976; Zentgraf, 1976; Zentgraf and Wapner, 1976) and zones of irreparable cell death (Gardner *et al.*, 1962, 1964; Eftis and Gardner, 1964). These are much more spatially mapped

CHAPTER THREE

The expression and possible roles of *muscle segment homeobox* genes, *Msx1* and *Msx2*, during mouse and chicken limb development: their potential for functional redundancy

Introduction:

The generation, by homologous recombination, of mice which were homozygous for a mutation in *Msx1*, was provocative because these mice showed no abnormal limb phenotype (Satokata and Maas, 1994). One possible explanation for this observation is functional redundancy between *Msx1* and *Msx2*, which is also expressed in the ectoderm and mesoderm of the developing limb bud (Coelho *et al.*, 1991a). To evaluate whether this explanation is plausible, one would need to know whether *Msx2* is expressed in the cells which express *Msx1* at each successive stage of limb development. To date however, there has been no comprehensive, detailed description of *Msx* gene expression in the mouse limb with which to make this evaluation.

A knowledge of the expression patterns of the *Msx* genes is fundamental to an understanding of their roles in the development of the embryo. Previous analyses of the expression of the genes has been limited by the *in situ* technique on tissue sections, which restricts the number of embryos which can be processed and makes a three-dimensional reconstruction of expression patterns difficult. A series of whole-mount *in situs* were performed with Liz Graham and Lesley McGinnis, between 1992 and 1994 using *Msx* probes on mouse and chicken limbs.

I analysed the expression of *Msx1* and *Msx2* in the mouse limb to investigate the degrees to which the expression patterns of *Msx1* and *Msx2* overlap. Expression of the chicken homologues (Suzuki *et al.*, 1991; Yokouchi *et al.*, 1991) was also reanalysed for two reasons. Firstly, although much information has been published piecemeal about the expression of the genes in the chicken limb, there has been no comprehensive stage-specific analysis, and this has lead to some confusion and inaccuracy in the literature. Secondly, the history of classical embryological experiments on the chicken limb has lead to operational identification of regions such as the Zone of Polarising Activity (ZPA) (MacCabe *et al.*, 1972; Honig and Summerbell, 1985), Progress Zone (Hornbruch and Wolpert, 1970) and zones of apoptotic cell death (Saunders Jnr., 1962, 1966; Fallon and Saunders, 1968). These are much more accurately mapped

than in the mouse. It is therefore possible to relate patterns of *Msx* expression to these areas during limb development in the chicken, and to make predictions about the functions of the genes. These predictions might be tested genetically in mice.

I analysed limbs during the early pattern forming stages of development, from their inception (stage 16 chickens, E9 mice) until chondrification of the digits (stage 31 chickens, E14 mice). At least 10 limbs were examined for every stage of development.

My observations showed that *Msx* gene expression in the limb is not restricted to any one cell type, and does not correlate with any single developmental process. There are clear differences between the expression patterns of the two genes, and it is possible therefore that there are some cells which express *Msx1* but not *Msx2*. The differences however are largely quantitative, and the broad similarity between the expression pattern of the genes argues that a large degree of functional redundancy would be possible.

There are minor differences between the expression of the genes in mice and chicken limbs of equivalent developmental stages, and between fore- and hind-limbs. These differences may contribute to the different shapes of the limbs.

Limb staging:

Mouse:

I found it useful to divide mouse limb buds into developmental stages according to the criteria set out by Wanek *et al.* (1989). By this assessment, limbs are divided into 15 developmental stages based on visible morphological features. Fore-limbs and hind-limbs are assigned to the same developmental stage when they reach similar shapes, even though the fore-limbs reach any given stage about half a day in advance of the hind-limbs. I have abbreviated these limb stages W1-W15.

Chicken:

Chickens were staged according to the criteria of Hamburger and Hamilton (1951)

Results:

Msx1 in the mouse:

Lateral plate mesenchymal cells of the limb field proliferate and form, with the overlying ectoderm, the developing limb bud. The fore-limb develops approximately half a day in advance of the hind-limb. This lag between fore- and hind-limb development is maintained subsequently, but fore- and hind-limbs of equivalent developmental stages show similar *Msx* expression patterns.

In situ data on tissue sections has previously shown *Msx1* expression in the lateral plate mesoderm in a domain which includes the operationally defined limb fields of the Wolfiann ridge (Davidson and Hill, 1992).

Msx1, but not *Msx2*, is also expressed in cells at the ventral margin of the dermamyotome which give rise to the limb muscles (see Fig. 3.5).

Whole-mount *in situ* hybridisation shows that with the upregulation of *Msx* genes in the limb bud at around E9, the bud becomes an island of *Msx* expression, adjacent to the non-expressing tissue of the body wall.

In this early stage (W1), prior to the morphological differentiation of the apical ectodermal ridge (AER), *Msx1* transcripts are detected throughout the limb bud mesenchyme (Fig. 3.1A). As the limb bud grows, due mainly to the mitotic activity of the distal cells (the Progress Zone), *Msx1* expression becomes restricted to the marginal mesenchyme of the limb, forming an anteriorly-biased arc of gene expression at stages W2 and W3 (Figs. 3.1B,C; 3.3A). The anteriorly-biased domain of marginal mesenchymal expression is maintained as the limb grows distally (W4). There is a broad, intense domain along the anterior margin of the limb mesenchyme, merging into a weaker, narrower, domain subjacent to the distal AER and a broader domain along the proximal-posterior margin (Figs. 3.1D, 3.3B). Transection of the whole mount limbs showed that the expression domain has no dorso-ventral bias (Fig. 3.1Da). As the digit plate becomes distinguishable (W5), expression is lost in the most posterior-proximal limb mesenchyme (Fig. 3.1E; 3.3C). Later (W6), expression becomes restricted to the subridge marginal mesenchyme of the autopodium in a symmetrical arc which extends only a little way into the zeugopodium (Fig. 3.1,F).

Msx1 transcripts are not abundant in the limb ectoderm. Prior to formation of the AER, *Msx1* expression is detectable in all but the most dorsal ectoderm (Fig. 3.1Aa). At the earliest stages of the AER, (~W3), expression is detectable throughout this structure (Figs. 3.1C; 3.3A (arrow); staining is weaker than that seen in the subjacent mesenchyme. *Msx1* is expressed, though barely detectable, in the ridge from W4

onwards; some of the most distal cells which cap the AER (Kelly and Fallon, 1981) are labelled. There is no other ectodermal expression.

From W7, apoptosis occurs in the mesenchyme between the chondrifying digits - producing recognisable fingers and toes. Coincident with the initiation of apoptosis in the digit plate, *Msx1* is expressed in these areas in a pattern which clearly demarcates the areas occupied by the forming digits. Condensation first occurs in digits 3 and 4 at stage W7, and it is between these that *Msx1* expression is first seen (Figs. 3.1G; 3.3D). At W8, expression is seen in all the apoptotic zones of the autopod (Fig. 3.1H). *Msx1* expression is maintained in the most distal marginal mesenchyme throughout. A transverse section of the limb reveals that the interdigital expression is genuinely in the mesenchyme where cells are dying, between the chondrifications. Not all the cells which express *Msx1* will die; there is expression in the perichondrium of the phalanges and in some of the prospective soft tissue of the digits (Figs. 3.1I,J; 3.3E). *Msx1* transcripts were last detected by whole-mount techniques in the soft tissue at the distal tips of the digits at W12 (Figs. 3.1K; 3.3F).

***Msx2* in the mouse:**

Msx2 is also expressed in the limbs during and subsequent to their inception. *Msx2* transcripts are detected in the ectoderm of the limb at stage W1 (Figs. 3.2A; 3.4A). It is coexpressed strongly with *Msx1* in all but the most dorsal ectoderm (Fig. 3.2Aa). Prior to formation of the AER, the epithelial expression becomes more distally restricted (Fig. 3.2Ba) and is expressed only in the AER upon its induction. At these early stages, *Msx2* expression is still detectable in the ectoderm outwith the limb. *Msx2* is expressed strongly throughout the ridge during stages W3, W4 and W5, more weakly during W6 and W7, at which stage the ridge starts to regress (Figs. 3.2C-H; 3.4B,C,D). *Msx2* is still detectable in the distal ectoderm of limbs of stages W8 and W9, albeit weakly.

Msx2 is also expressed in the mesenchyme of the developing bud. At W1 expression is confined to the ventral-anterior limb mesenchyme (Fig. 3.2A) - much weaker than expression in the overlying epithelium and weaker than *Msx1* mesenchymal expression. At W2 transcripts are found in a band in the anterior 2/5

of the limb mesenchyme, overspilling slightly into the mesoderm anterior to the limb bud (Fig. 3.2B). W3 sees the inception of a posterior-proximal expression domain, which is maintained and extends along the posterior marginal mesenchyme (Fig. 3.2C). Hence, by W4, mesodermal *Msx2* expression is contained within the region of *Msx1* expression (Figs. 3.2D; 3.4B). Expression in the distal mesenchyme is restricted to the cells most directly subjacent to the AER (in agreement with the results of Davidson *et al.* (1991)). There is no dorso-ventral bias to mesodermal expression at this and subsequent stages (Fig. 3.2Da).

During stages W5 and W6, *Msx2* expression in the mesoderm becomes restricted to the digit plate, in a pattern which reflects the changes occurring simultaneously in *Msx1* (Figs. 3.2E,F; 3.4C,D). Expression is broadest at the junction of the autopod and zeugopod where cell death is thought to occur (Figs. 3.2F, 3.4C). Around the distal margin of the digit plate, *Msx2* is just detectable in the most marginal mesenchymal cells subjacent to the AER (Fig 3.3.2F, 3.4D arrows).

There is also a region of expression at stages W6 and W7 high up in the anterior shoulder region - an area where *Msx1* expression is also seen. This domain includes cells which will contribute to the brachial limb muscles.

Like *Msx1*, *Msx2* is expressed interdigitally from W7 in a domain which is coincident with that of *Msx1*, but is not as extensive - *Msx2* is expressed in the perichondrial tissue and in the zones of cell death (Figs. 3.2H,I,J; 3.4E). There is detectable expression in the metacarpal/tarsal regions of stage W8 and W9 limbs (in the mesenchyme around the chondrifications and in a small region of ectoderm at the back of the hand), where *Msx1* expression has not been observed (Fig. 3.4E). By W12 this is visible as weak expression in the ventral fatty tissue in the preaxial regions of the fore-limbs. There is also weak expression in the soft tissue around the distal phalanges of fore- and hind-limbs, in common with *Msx1* (Figs. 3.2K; 3.4F).

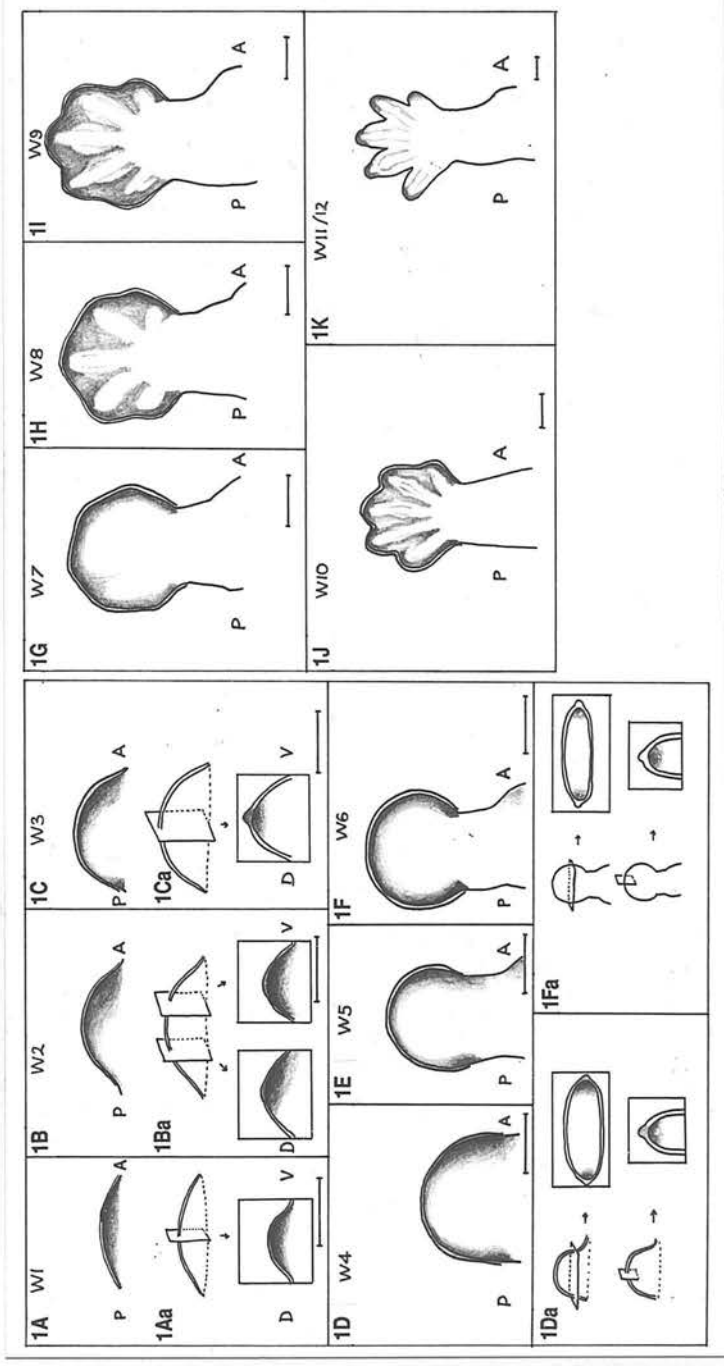


Figure 3.1: Expression of *Msx1* in mouse limbs between stages W1 and W12 (Limb stages from Wanek *et al.*, 1989). Anterior is to the right in all cases, except for dorso-ventral views (suffixed 'a') in which ventral is to the right. Scale bar represents 500µm. See text for details. Abbreviations: A - anterior; P - posterior; D - dorsal; V - ventral. These pictures represent whole limbs, and were drawn from actual specimens. They are a summary of the text description

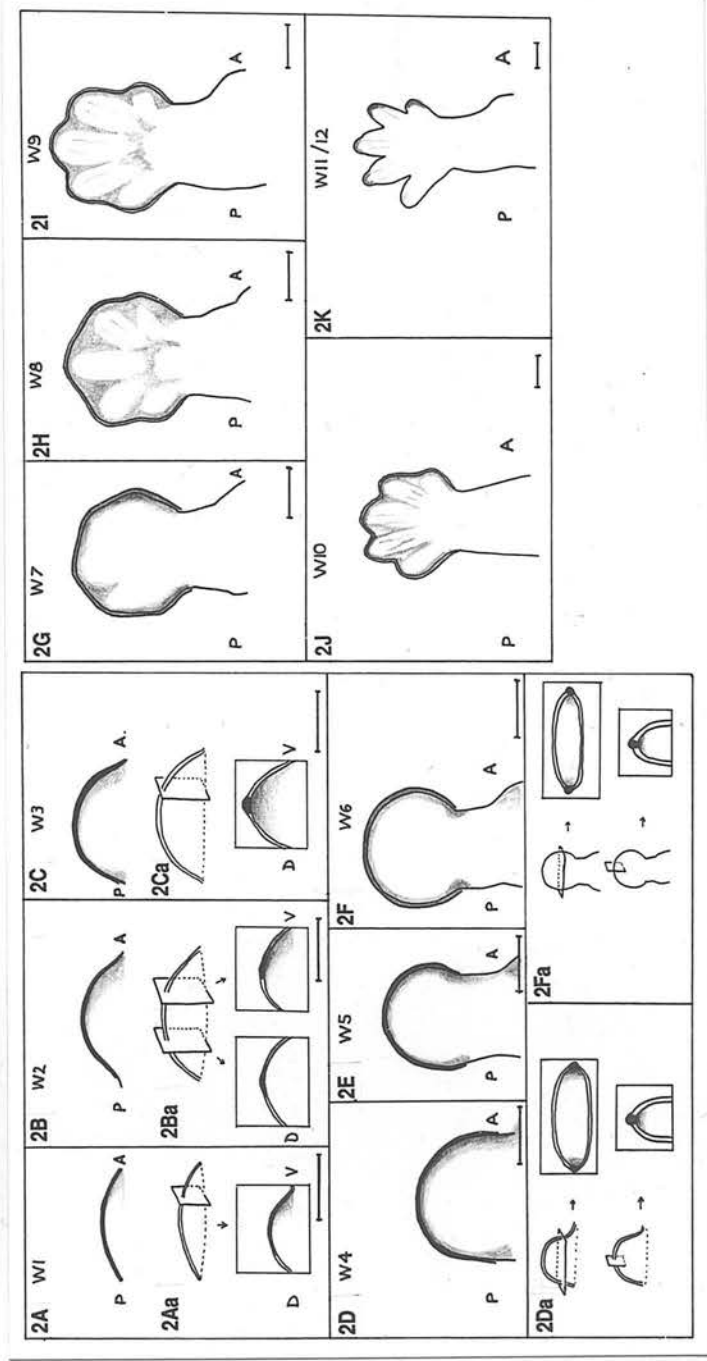


Figure 3.2: Expression of *Msx2* in mouse limbs between stages W1 and W12 (Limb stages from Wanek *et al.*, 1989). Anterior is to the right in all cases, except for dorso-ventral views (suffixed 'a') in which ventral is to the right. Scale bar represents 500µm. See text for details.
 Abbreviations: A - anterior; P - posterior; D - dorsal; V - ventral.
 These pictures represent whole limbs, and were drawn from actual specimens. They are a summary of the text description.

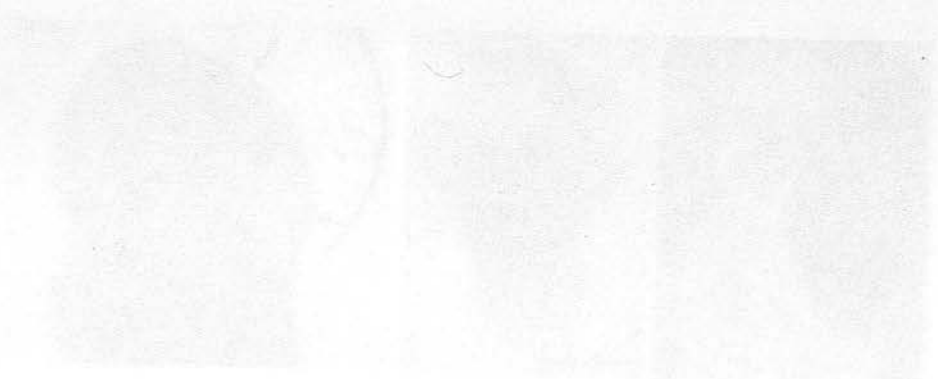
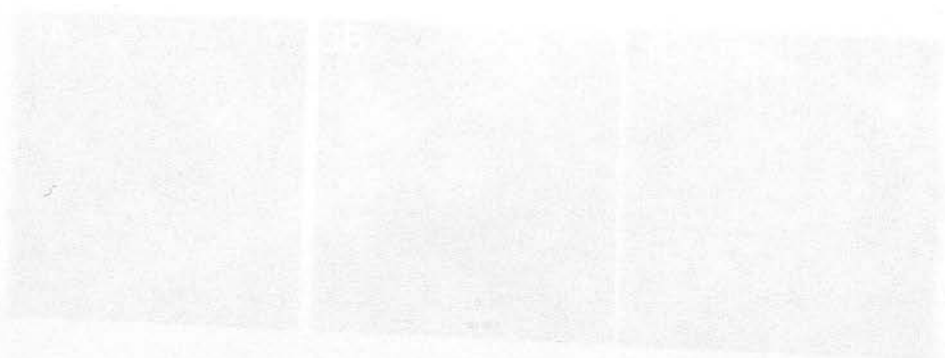


Figure 3.3: Expression of *Msx1* in mouse limbs stage W1 (A), W4 (B), W5 (C), W7 (D), W10 (E) and W12 (F).

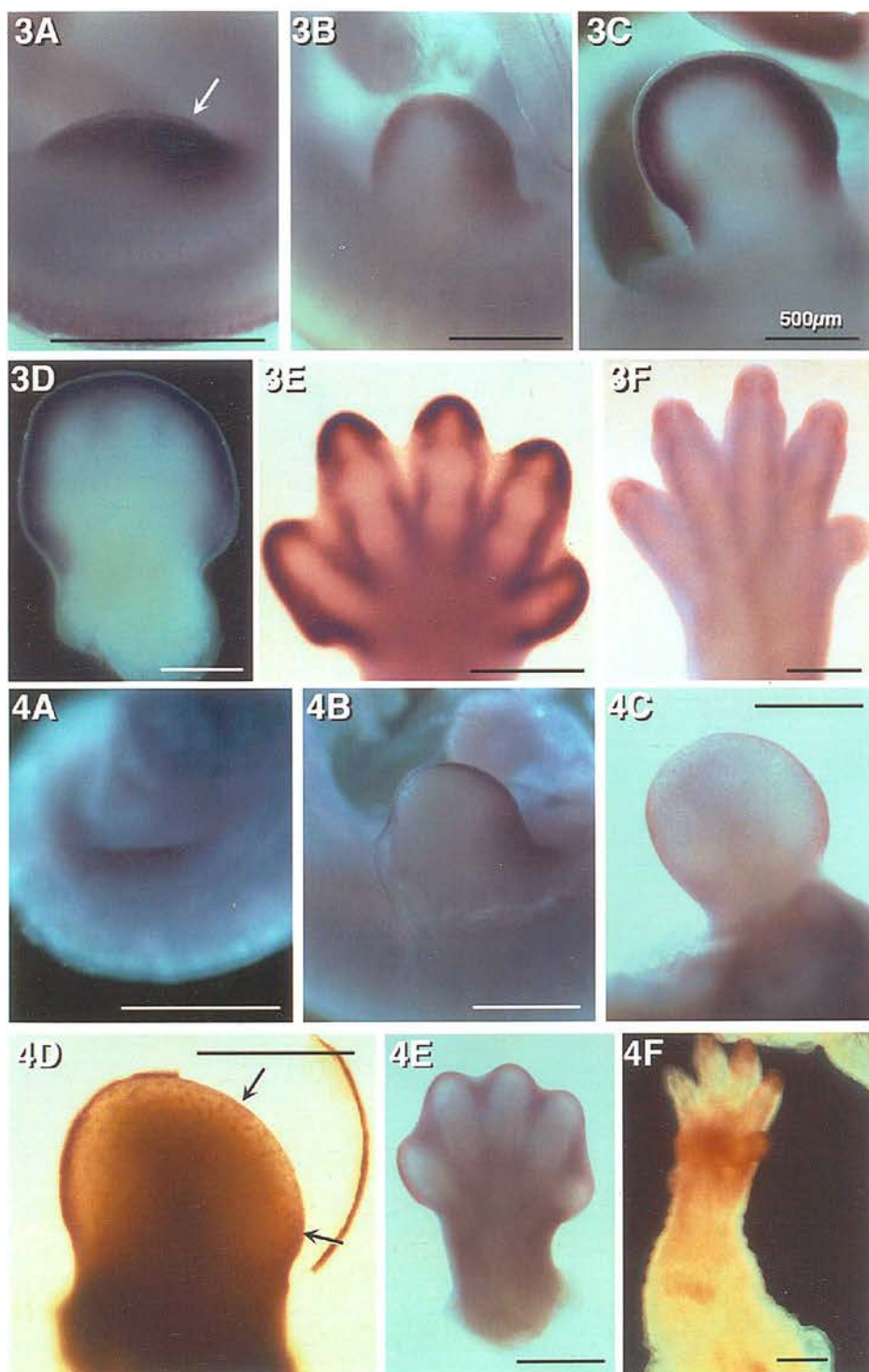
Anterior is to the right; the scale bar represents 500µm.

The arrow in 3.3A shows weak staining in the limb ectoderm.

Figure 3.4: Expression of *Msx2* in mouse limbs stage W1 (A), W4 (B), W5/6 (C,D), W9 (E) and W12 (F).

Anterior is to the right; the scale bar represents 500µm. 3.4F presents a ventral view of the fore-limb, showing staining in the soft tissue of the palm.

The AER of the limb in 3.4D has been partially removed - it is strongly stained, but staining is also visible in the underlying mesenchyme (arrows). See text for details.



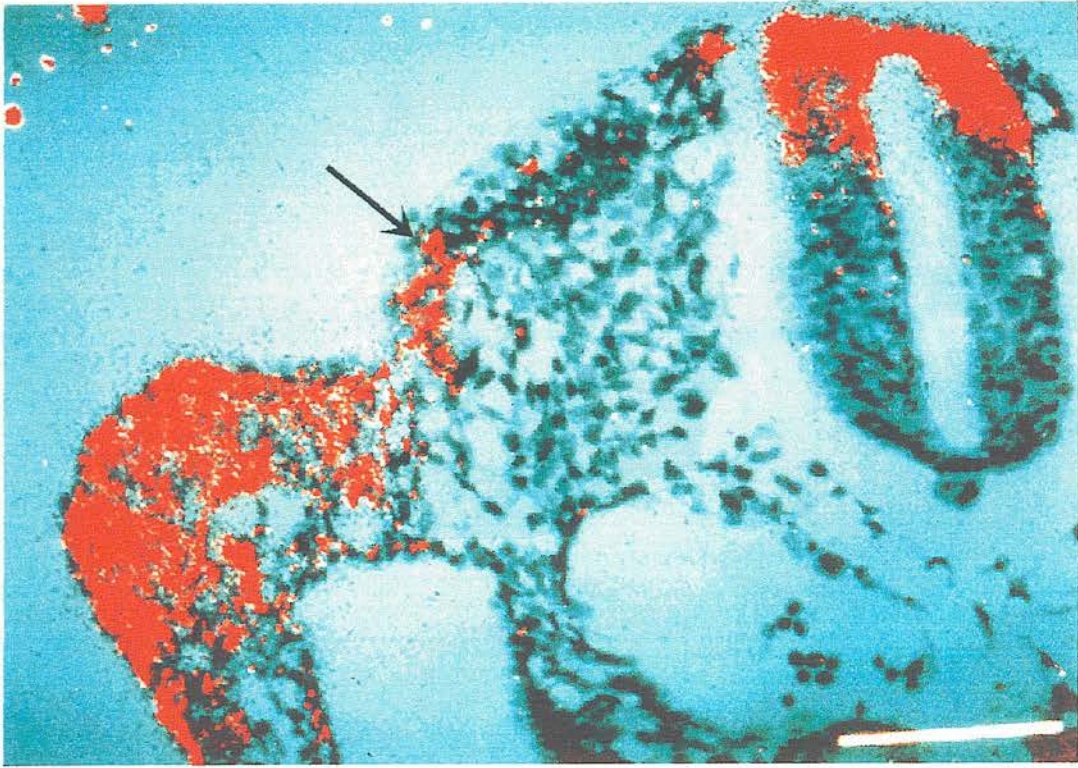


Figure 3.5: Computer-enhanced radioactive *in situ*, using *Msx1* probe, of a transverse section at forelimb level of an E10 mouse. Signal is seen in the dorsal neural tube (top right), the limb mesenchyme (bottom left) and the cells of the ventrolateral dermamyotome (arrow). Scale bar = 100 μ m. This slide was provided by Duncan Davidson.

***Msx1* in the chicken:**

Msx1 mRNA is abundant in the limb mesenchyme of the chicken at stages 16, 17 and 18 (Figs. 3.6A,B). At stage 19, mesenchymal expression is biased anteriorly in the fore-limbs (Figs. 3.6C; 3.8A), with strongest, broadest expression in the mesenchyme of the anterior half of the bud (in the hind-limbs, the gene is still expressed uniformly at this stage, and the anterior-distal bias is not seen until stage 21).

By stage 21, *Msx1* transcripts can be detected in an anteriorly-biased arc in the mesenchyme around the distal margin of the limb (Fig. 3.6E). Expression is narrower and broader in the more posterior-proximal areas of the bud, until stage 22 when it starts to rally (Figs. 3.6F; 3.8B). This pattern is maintained as the limb grows distally, so that by stage 23, expression looks very like that of *Msx1* in a mouse limb of equivalent developmental stage (W4) (Fig. 3.6G, cf. Fig. 3.1D).

Msx1 is expressed in the ectoderm of the early chick limb bud although, as in the mouse, expression is always weaker than in the mesenchyme. Transcripts are detected in the ectoderm of the stage 17 limb bud, with a ventral bias, and become progressively more distally restricted to the AER at stages 19 and 20 (Figs. 3.6Aa,Ba,Ca). Expression is also seen in the cells just adjacent to the ridge, which can be incorporated into the ridge itself. However, this uniform ridge labelling is transitory; by stage 22 staining is seen only in the ridge at the anterior and posterior margins (Fig. 3.6F); this is a pattern which is maintained up to stage 26 at least - staining in the limb ectoderm overlaying the anterior and posterior mesenchymal domains of expression (Fig. 3.8C, arrow).

In the mesenchyme, between stages 23 and 24, the gene is downregulated in the most distal (Progress Zone) region of the bud, such that two discrete expressing regions are seen by stage 24 - strongly along the anterior margin of the bud and a smaller, weaker, area along the posterior margin (Figs. 3.6H; 3.8C). These domains include the anterior and posterior 'necrotic' zones respectively. There is no *Msx1* expression in the 'opaque zone', the zone of apoptotic cell death which will separate the two long bones of the zeugopod. At stage 25/26, the limb is morphologically distinguished into a digit plate and a jointed zeugopodium/stylopodium. *Msx1* expression is found down the whole anterior margin of the limb. It is also expressed in the posterior of the digit plate, extending a little into the zeugopodium (Figs. 3.8D; 3.6I,J). Between stages 26 and 28, in an analogous process to that which occurs in the mouse, *Msx1* becomes restricted to the marginal mesenchyme of the autopod (Figs. 3.6I-L). This requires re-initiation of the gene in the most distal regions of the bud, a process which occurs slightly earlier (stages 26/27) in the hind-limbs than in the fore-limbs (stages 27/28) (Fig. 3.6K). Expression is then initiated in the

apoptotic zones of the autopod, demarcating the outline of the chondrification of the digits by stage 29 (Figs. 3.6L, M, N; 3.8F). It is found in the interdigital regions where cell death is occurring, but rapidly fades out so that, after stage 33, virtually no mesenchymal expression is visible away from the perichondrium.

***Msx2* in the chicken:**

Msx2 expression is visible in the ectoderm of the nascent limb at stage 16. As in the mouse, it is detected in all but the most dorsal ectoderm (Fig. 3.7A, Aa). During stages 17 and 18, the domain becomes more distally restricted, such that at stage 19, *Msx2* transcripts are detected throughout the AER and nowhere else in the ectoderm (Figs. 3.7Ba,Ca; 3.9A).

Msx2 transcripts are just detectable in the limb mesenchyme at stage 18 but, coincident with the anterior bias in *Msx1* expression at stage 19, are found in the anterior 2/5 of the bud, and just reaching the distal tip (Fig. 3.7C). Expression of *Msx2* in the distal mesenchyme is never very strong, and soon fades such that by stage 22 there is an anterior domain of expression and, like *Msx1*, a newly-initiated posterior-proximal domain of mesenchymal expression (Fig. 3.7F).

The absence of *Msx2* expression distally is only a temporal difference between *Msx2* and *Msx1*, as by stage 24 and subsequently, when distal expression of *Msx1* is downregulated, the expression patterns of the two genes largely coincide (Fig. 3.7H, cf. Fig. 3.6H). By stage 24 the anterior mesenchymal expression is strongest in the zeugopodium and at stage 25 there is strongest expression in the zeugopod and shoulder/hip joints (Figs. 3.7I; 3.9D). There is also a region of expression at the junction of the zeugopod and autopod at the posterior margin - this lies within the equivalent *Msx1* expressing region (Figs. 3.7I,J; 3.9D, cf. Figs. 3.6I,J; 3.8D).

Between stages 19 and 23, *Msx2* is expressed at very high levels throughout the AER, and also more weakly in the marginal ectoderm down the anterior boundary of the limb. Ectodermal expression hereon is weaker, but detectable, at least until regression of the AER at stages 27/28 (Figs. 3.7C-L; 3.9A-E). At stage 26/27, *Msx2* is reinitiated distally in the digit plate of the legs, and by stage 27/28 both sets of limbs

show gene expression round the distal margin of the autopod. This is coincident with the distal reinitiation of expression of *Msx1* distally (Figs. 3.7K,L; 3.9E, cf. Figs. 3.6K,L; 3.8E). At stages 28/29, *Msx2* expression in the zeugopodium is downregulated as INZ expression is upregulated. During stages 29-31, expression is seen only in the apoptotic zones of the autopod. *Msx2* disappears from the digital webs before cell death is complete (Figs. 3.7M,N; 3.9F).



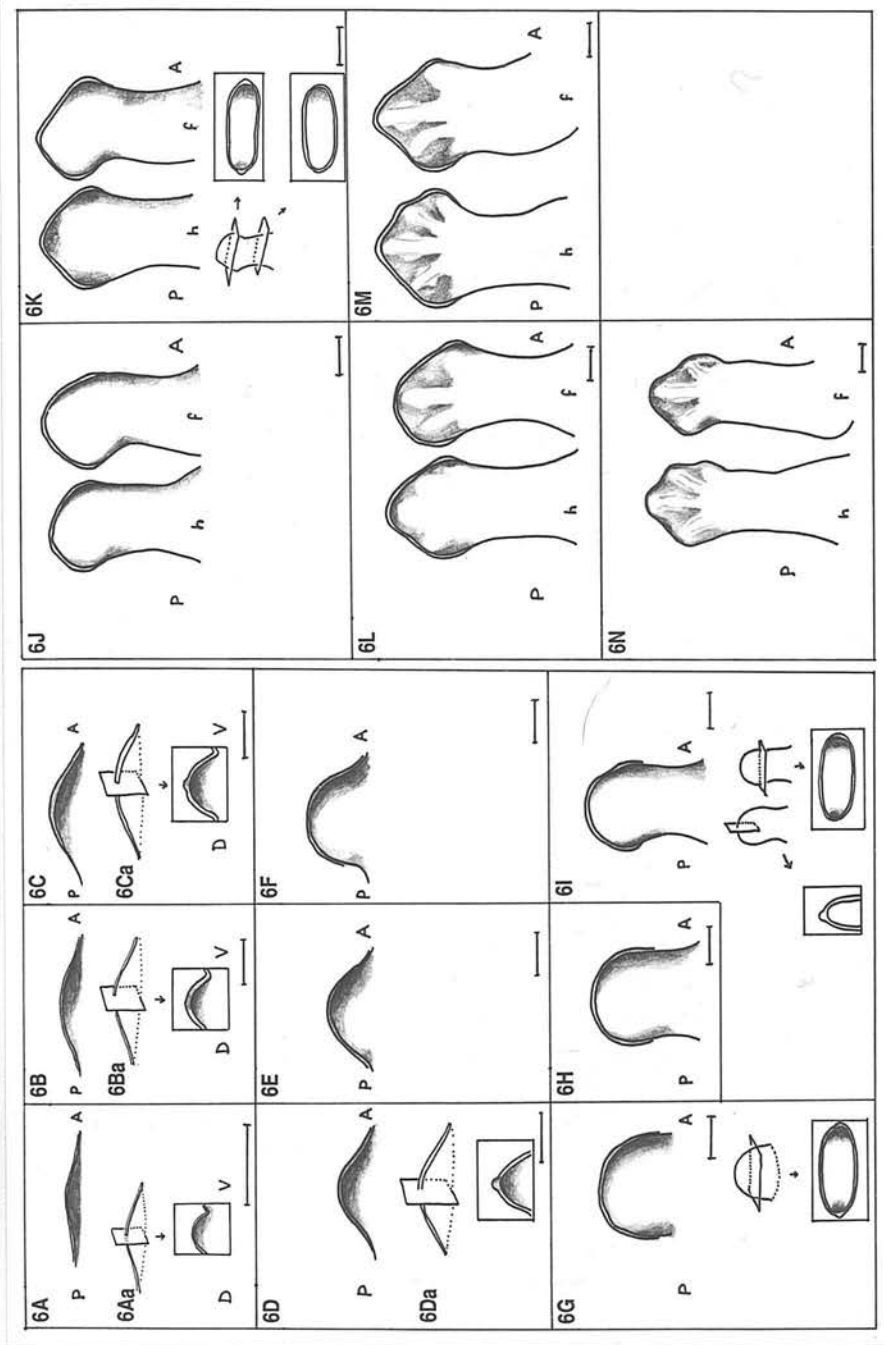


Figure 3.6:

Expression of *Msx1* in chicken limbs stage 16/17 (6A), 18 (6B), 19 (6C), 20 (6D), 21 (6E), 22 (6F), 23 (6G), 24 (6H), 25 (6I), 26 (6J), 27 (6K), 28 (6L), 29/30 (6M) and 31/32 (6N).

Anterior is to the right in all cases except for dorso-ventral views (suffixed 'a') in which the ventral side is toward the right. 6J-6N are of stages where the fore- and hind-limbs differ significantly in shape, and both limbs are shown.

These pictures represent whole limbs, and were drawn from actual specimens. They are a summary of the text description.

Scale bar represents 500µm. See text for details.

Abbreviations: h - hind-limb; f - fore-limb; A - anterior; P - posterior; D - dorsal; V - ventral.

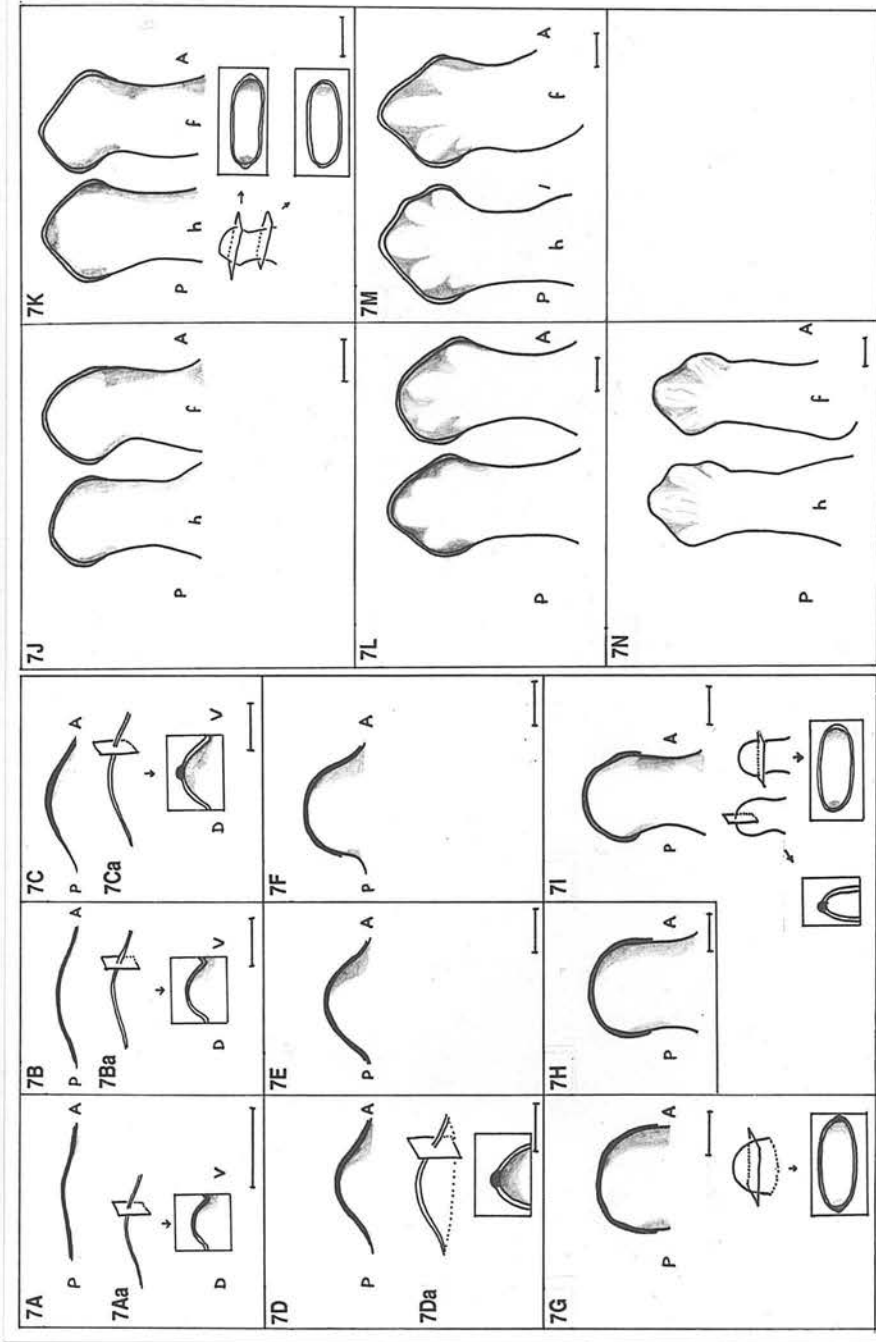


Figure 3.7:

Expression of *Msx2* in chicken limbs stage 16/17 (7A), 18 (7B), 19 (7C), 20 (7D), 21 (7E), 22 (7F), 23 (7G), 24 (7H), 25 (7I), 26 (7J), 27 (7K), 28 (7L), 29/30 (7M) and 31/32 (7N).

Anterior is to the right in all cases except for dorso-ventral views (suffixed 'a') in which the ventral side is toward the right. 7J-7N are of stages where the fore- and hind-limbs differ significantly in shape, and both limbs are shown.

These pictures represent whole limbs, and were drawn from actual specimens. They are a summary of the text description.

Scale bar represents 500µm. See text for details.

Abbreviations: h = hind-limb; f = fore-limb; A = anterior; P = posterior; D = dorsal; V = ventral.





Figure 3.8: Expression of *Msx1* in chicken limbs stage 19 (A), 22 (B), 24/25 (C), 26 (D), 27/28 (E) and 31 (F).

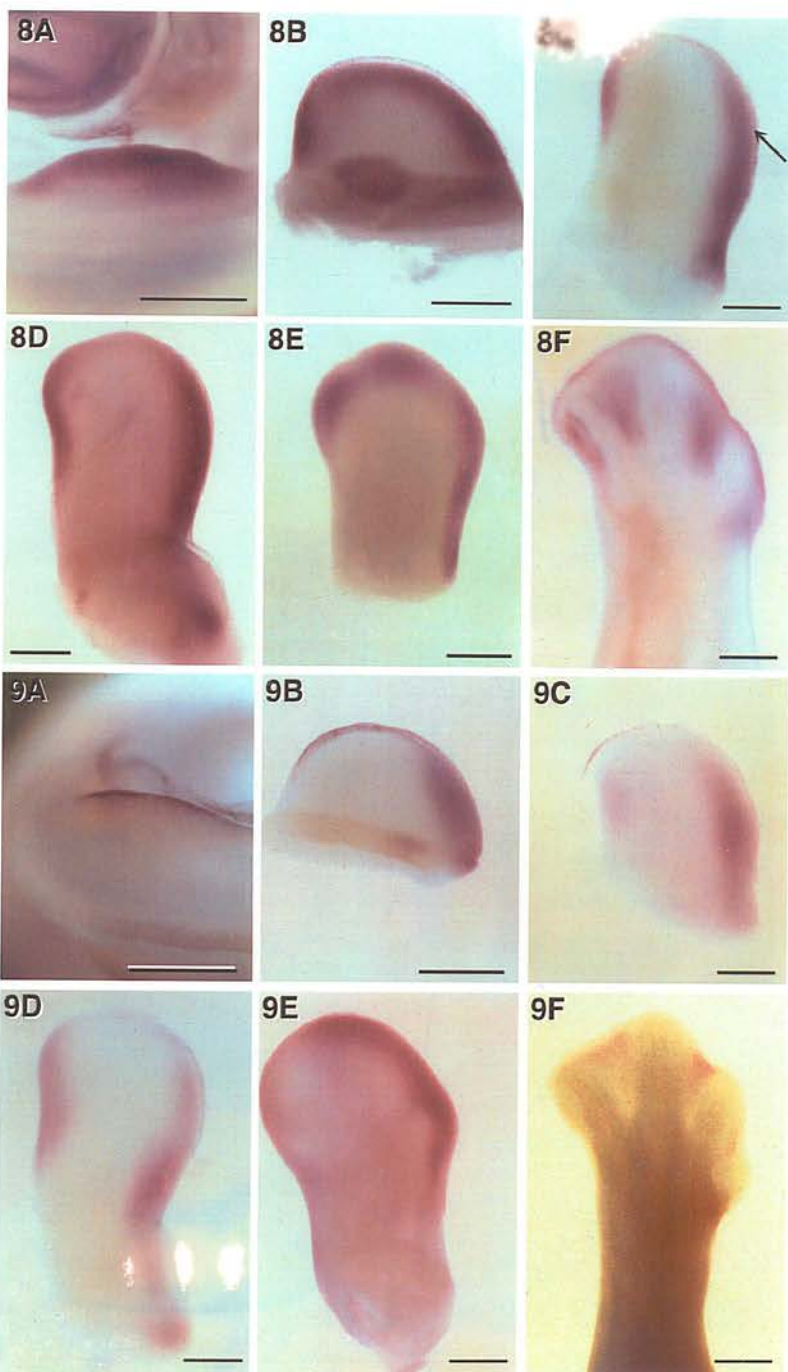
Anterior is to the right. Scale bar represents 500 μ m. All photographs show fore-limbs, except 8E, which is a hind-limb.

Staining is visible in the periderm of the AER of 8B. The arrow in 8C shows staining in the epithelium overlying the anterior domain of *Msx1* expression. See text for details.

Figure 3.9: Expression of *Msx2* in chicken limbs stage 19 (A), 21/22 (B), 24/25 (C), 26 (D), 28 (E), 31 (F).

Anterior is to the right. Scale bar represents 500 μ m. All photographs show fore-limbs, except 9F, which is a hind-limb. See text for details.





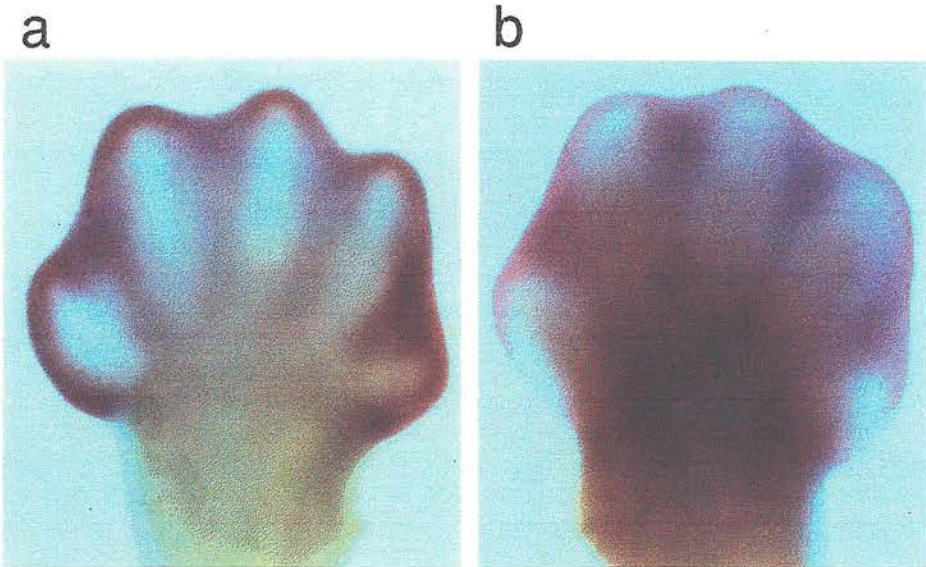


Figure 3.10: Expression of *Msx1* (left) and *Msx2* (right) in W9 mouse limbs.

Conclusions:

On the basis of this data it was possible to define separable domains of *Msx* gene expression in the limb, to correlate these with known developmental processes and hence to put forward possible functions for the genes. This analysis will be presented in Chapter 8. Whereas the expression pattern of the two genes are similar, they are not identical and it is not possible at this time to unequivocally state that *Msx2* could compensate fully for *Msx1* in the limb (see page 205 and Fig. 3.10).

In the immediate experimental sense, the analysis provided the first comprehensive description of *Msx* gene expression during the pattern-forming stages of limb development. I noticed a close correlation between the domains of *Msx* gene expression and the published reports of *Bmp4* expression in mice (Jones *et al.*, 1992; Hogan, 1994) and chicken limbs (Francis *et al.*, 1994) (AER, anteriorly-biased arc in the mesenchyme, then ANZ, PNZ and interdigitally, and in the perichondrium also)

Vainio *et al.* (1993) had shown that BMP4 was a candidate upstream regulating molecule for *Msx1* and *Msx2* in dental mesenchyme; I therefore performed the experiments described in Chapter 7 to show that BMP4 could induce *de novo* expression of *Msx* genes in limb mesenchyme *in vivo*.

CHAPTER FOUR

Analysis of the *Msx1* promoter Nco5' fragment.

Introduction:

In Chapter 1, I described how it is possible to investigate the control and function of genes by linking their promoter regions to a reporter gene such as *lacZ*, and studying the behaviour of the reporter construct in cells *in vitro* and *in vivo*. The pH7lacT and the pH7lacΔ3' constructs (see pages 67-69) were available which would allow me to perform experiments with an *Msx1* promoter region (the 4.7kb *Nco1* fragment) in frame with *lacZ*. The pH7lacT construct, being the larger of the two, was more likely to contain all the promoter sequences and processing signals necessary to mimic the expression of endogenous *Msx1*. In the first instance, it was decided to use this construct in experiments aimed at finding the 'AER response element' - sequences in the promoter which were necessary for the *Msx1* gene to be expressed in the distal mesenchyme of the Progress Zone in response to the AER signal.

The construct was to be introduced into cells, then cells introduced into the limb - either distally under the AER of a stage 20 chick wing, or proximally into the stage 22 wing (grafting as performed by Davidson *et al.* (1991).

If it could be shown that in the former case (distal grafts), *lacZ* was expressed from the promoter, but not in the latter case (proximal grafts), then the next step would be to cut down the promoter, repeating the experiments until the AER response was lost (or any other perturbation of gene expression occurred).

This experimental procedure has certain advantages over the more conventional transgenic approach to promoter dissection. The first of these is speed. By introducing DNA directly into cells, e.g. by microinjection, it should be possible to analyse expression of a construct within forty-eight hours, compared to a minimum two weeks to produce a transgenic embryo. The second advantage is that it is possible to analyse expression of the construct in a large number of grafts (compared to the number of transgenic embryos which can be produced in a given time). These two advantages combine to increase the resolution of the promoter dissection which can, in theory, be performed.

This chapter describes the development of a technique for introducing the constructs into cells, and the cells into limb buds in an attempt to show position-specific induction of *lacZ*. It was originally intended to introduce the constructs into cultured limb mesenchyme, then graft this transfected mesenchyme into chicken limbs. Initial attempts to introduce constructs into primary limb cells by microinjection were unsuccessful, but the experiments were continued using 10T^{1/2} cells. Although the techniques worked

well and the experiments were possible, a convincing AER response was never demonstrated. One of the reasons for this was a problem with the ability of 10T^{1/2}s to express *Msx* genes, but experiments performed in subsequent chapters provide other reasons.

The experiments in this chapter provide strong evidence for regulatory regions, affecting the expression of *Msx1*, which are localised downstream of the transcription start site.

To demonstrate that the *Msx1* promoter was functional it is necessary to show that:

- (i) Cells could be transfected with plasmid DNA and that they were subsequently capable of expressing the injected gene.
- (ii) Injected cells in which *Msx1* could be overexpressed into the developing chicken limb, and that they would survive to a level that allowed the transcription of the experiment.

Experiment 4.01

The results of Davidson et al. (1994) suggested that expanded mesenchymal cells can respond to signals other than that released by the AER:

Altogether these results suggest that the AER is not the only source of AER signals.

Supporting the results of Davidson et al. (1994) we performed experiments and taken from the proximal end of an E11.5 mouse limb and grafted ectodermally under the AER of a stage 39 chick wing or proximally under the mesoderm (Figure 4.1).

After overnight culture explants were fixed, processed and sectioned for ³⁵S in situ hybridisation using *Mx1* probes. Chicken *Mx1* probes were used to achieve alignment to the mouse gene.

14-17/12 grafts, from 2 experiments, showed *Mx1* expression only distally in mesenchymal tissues to the AER (Figure 4.2A,B,C). 2 grafts were directly adjacent to the AER and expressed *Mx1* throughout, 7 were only partly within the chicken *Mx1* domain and expressed *Mx1* only distally. 3 negative grafts were outside the chicken *Mx1* domain.

These results are the same as those obtained by Davidson et al. (1994) and Bailey et al. (1997).

Part 1:

Introducing DNA to cells by direct nuclear microinjection:

It was desirable to avoid potential silencing of introduced genes by minimising the transfection time before cells were introduced into the limb. Microinjection was chosen as the fastest means of introducing DNA into a large number of cells.

To demonstrate that the planned experiments were feasible, it was necessary to show that:

- (i) Cells could be injected with plasmid DNA and that they were subsequently capable of expressing the introduced gene;
- (ii) Injected cells in tissue culture could be transferred into the developing chicken limb, and that they would survive in a healthy state during the time-course of the experiment.

Experiment 4.01

The results of Davidson *et al.* could be repeated and extended - mesenchyme cells can respond to signals other than that released by the AER:

Mouse limb mesenchyme can reinitiate gene expression if placed under the AER:

Repeating the results of Davidson *et al.*, de-epithelialised mesenchyme was taken from the proximal region of an E11.5 mouse hind-limb and grafted either distally under the AER of a stage 20 chick wing or proximally under the dorsal epithelium (Fig. 4.1).

After overnight culture, embryos were fixed, processed and sectioned for ^{35}S *in situ* hybridisation, using *Msx1* probes. Chicken *Msx1* probes were used in sections adjacent to the mouse gene.

In 12/12 grafts, from 2 experiments, mouse tissue expressed *Msx1* only distally in mesenchyme subjacent to the AER (Figure 4.2A,B,C). 2 grafts were directly subjacent to the AER and expressed *Msx1* throughout, 7 were only partly within the chicken *Msx1* domain and expressed *Msx1* only distally. 3 negative grafts were outside the chicken *Msx1* domain.

These results are the same as those obtained by Davidson *et al.* (1991) and Brown *et al.* (1993).

Mouse tissue can reinitiate *Msx1* and *Msx2* if placed in *Msx* expressing domains underneath the anterior, non-ridge epithelium of the limb:

Mouse limb mesenchyme was isolated as before. It was grafted into the anterior margin of the stage 22 chick wing (Fig. 4.1). After overnight culture, grafts were fixed, sectioned and probed by ^{35}S *in situ* with *Msx1* and *Msx2* probes.

9/9 grafts, from 2 experiments, were positive for both *Msx1* and *Msx2* only within the appropriate *Msx*-expressing regions of the chicken along the anterior margin of the limb (stg 26/27) (Figure 4.2 D-I).



Figure 4.1
Experiment 401
The results of Davidson et al. (1993) can be repeated and expanded. The grafts with appropriate expression in experimental procedure.

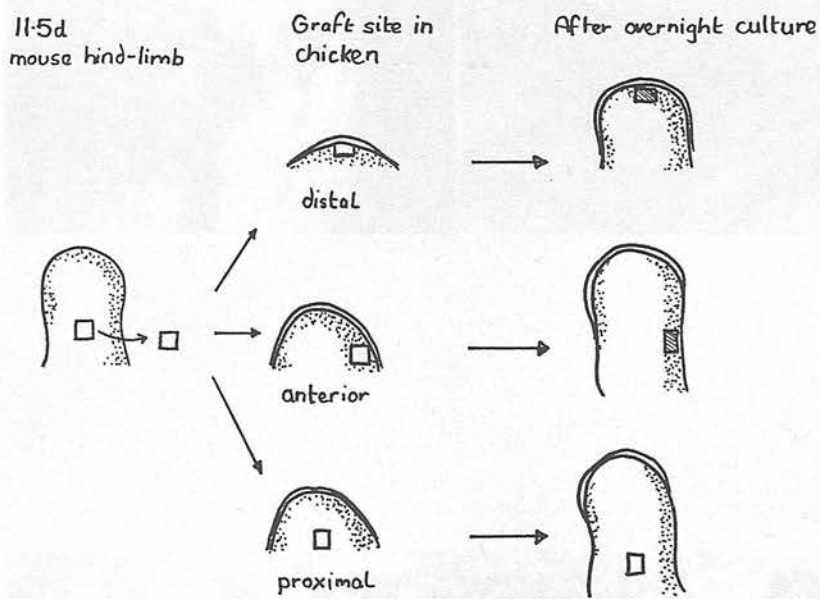


Figure 4.1:

Experiment 4.01

The results of Davidson *et al.* (1991) can be repeated and extended. Diagrammatic representation of experimental procedure.

Figure 4.2:

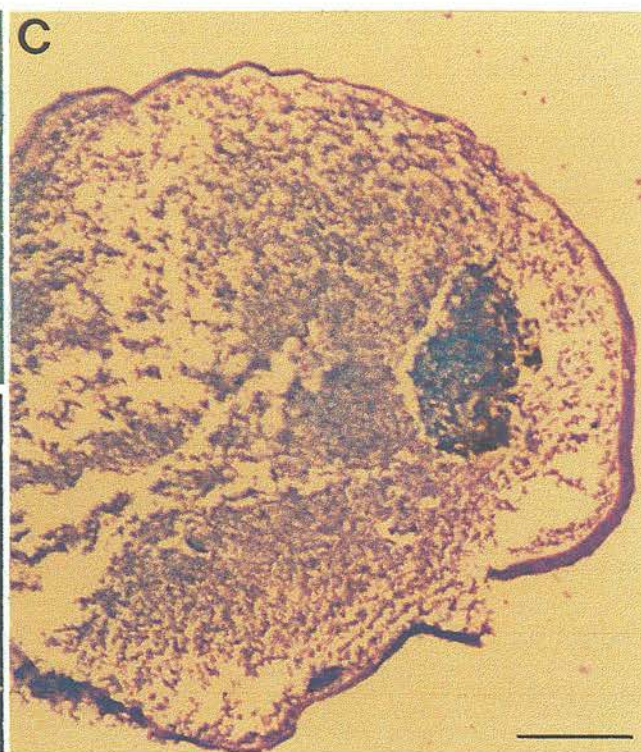
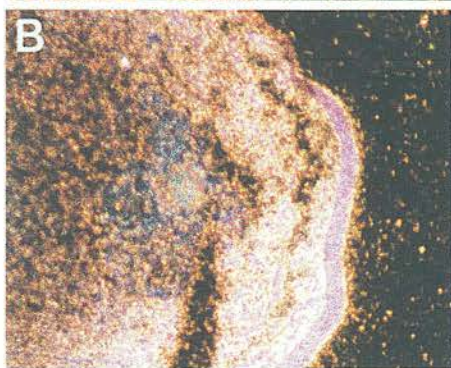
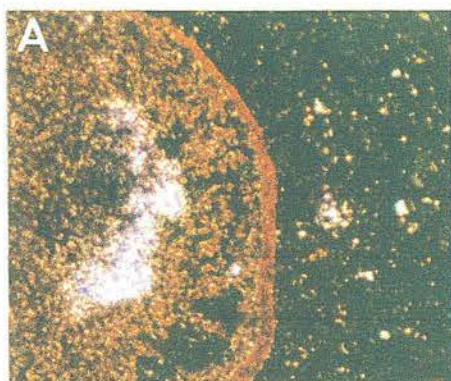
E11.5 proximal hind-limb mesenchyme grafted into chicken wings overnight

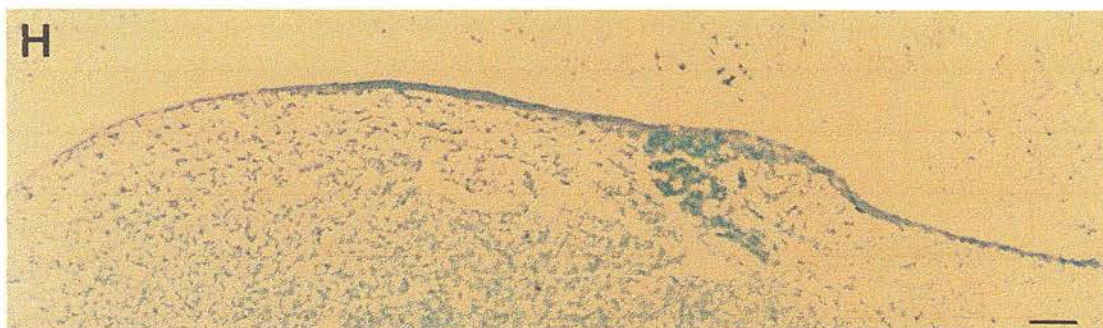
A,B,C - Distal graft underneath the AER. *In situ* hybridisation using mouse (A) or chicken (B) *Msx1* probe. C is bright field.

D,E,F,G - Anterior graft. D is bright field; E, chicken *Msx1*; F, mouse *Msx1*; G, mouse *Msx2*.

H,I, J - Anterior graft. H is bright field; I, chicken *Msx1*; J, mouse *Msx1*

Scale bar represents 50µm





Expression of constitutively expressed reporter constructs in injected cells:

Having shown that the grafting technique was working, it was now necessary to show that the injections could be performed, and that this was compatible with grafting.

Choice of cells for injection:

Primary limb mesenchyme cells would be the cells of choice, because these are closest to the *in vivo* situation for the purpose of response to extrinsic signals in chicken limbs. (One of the reasons for choosing microinjection as a means of transfection was that it could be used on primary cells). Cell lines were more convenient, as they required no special preparation to obtain a monolayer for microinjection. Of the large numbers of cell lines available, 10T^{1/2}s were chosen as a fibroblastic line which could respond to extrinsic signals to undergo differentiation (see page 66). H3M cells were chosen as a possible neural crest-derived cell line which should therefore be able to express *Msx* genes (page 66). COS-7 (immortal monkey kidney) cells express the SV40 large T antigen, and were chosen as positive controls in case the introduced constructs were expressed at very low levels - they amplify the introduced pSV β plasmid up to 100000-fold.

Experiment 4.02

Injected cells can express pCMV β and pSV β , but cell death is significant in some cases:

Chicken and mouse limb mesenchyme cells were separately plated out as described in Chapter Two. After twenty-four hours, cells growing as a monolayer were microinjected with pCMV β or pSV β . After a further 18 hour culture, the cells were fixed and stained with X-Gal. Less than 1% of 1600 mouse cells and 1400 chicken cells showed β -Gal activity. This was probably mainly due to cell death, as most cells detached from the substrate within a few hours of microinjection, leaving holes in the monolayer. It was impossible to remove the remaining cells from the dish in sheets suitable for grafting. The experiments described below shows that there was nothing intrinsically wrong with the microinjection protocol, as other cells can survive injection.

Control experiments were performed, injecting 10T^{1/2}, COS-7 and H3M cells with either pCMV β or pSV β .

Cells were injected with either pCMV β or pSV β , then returned to 37°C for 18 hours, after which they were fixed and stained with X-Gal to assay β -galactosidase activity.

As with primary mesenchyme cells, H3M cells did not survive injection procedure. Cells detached from the substrate within a few hours of microinjection. It is uncertain why this was so, and it may be possible in future to optimise the protocol and increase survival.

Expression of reporters in surviving cells:

Cells which expressed *lacZ* were stained intensely blue. Uninjected cells were unstained, and there was no endogenous activity detected in any of the cell lines used. Hence all cells were either unequivocally 'blue' or 'clear' - there was no middle ground of weakly stained cells (as expected from the nature of the X-Gal staining, which amplifies β -gal activity to give a strong blue signal).

More than 5000 $10T^{1/2}$ cells were injected during these experiments (and subsequently as controls during later experiments). Concentrations of injected DNA from 0.5 μ g/ μ l to 10ng/ μ l were used. There was no difference in average frequency of expression suggesting that plasmid DNA was present in excess, and that higher concentrations of DNA are not toxic.

With both pCMV β and pSV β , the usual rate of successful gene expression in $10T^{1/2}$ s was 7-12% i.e. if I attempted to inject a square of 500 cells, subsequent staining would show that 35-60 cells were expressing the constitutive reporter. This figure remained remarkably constant throughout subsequent experiments and probably represented the maximum transformation rate I could achieve using this technique.

The transfection rate was not 100% for a number of reasons. Many cell nuclei would not be hit by the needle, whereas others would be too badly damaged to survive. I therefore concluded that the 7-12% transformation rate represents constitutive gene activity in all surviving cells that were successfully injected.

Similar optimal expression was seen in over 2000 COS-7 cells injected with pCMV β and pSV β .

Both $10T^{1/2}$ cells and COS-7 cells could be cultured for at least 72 hours after injection and still show strong blue staining when fixed after this time. Hence, even allowing for the reported perdurance of β -Gal, expression of the introduced gene would continue in injected cells during the time course of grafting experiments.

Experiment 4.03:

10T^{1/2} and COS-7 cells survive grafting procedures and 24 hours in the limb:

A series of grafts was then performed of uninjected 10T^{1/2} cells and COS-7 cells into the developing wing bud of stage 20-21 chickens. As described in Chapter 2, 1.5mm squares of ~3000 cells were removed from the dish as a monolayer and allowed to form balls in non-tissue culture dishes overnight. They were grafted into chick wing buds and left for 24 hours *in ovo* before fixing in 4% paraformaldehyde. The graft at fixation was still visible in the limb as a discrete ball of cells.

These limbs were then wax-embedded and sectioned to be examined under the light microscope. This revealed that both 10T^{1/2} and COS-7 cells survived the grafting procedure well. The grafts were discrete balls with little or no integration into the limb. Cells appeared healthy; they showed general morphology similar to cells in culture, with cells on the outside of the graft being more flattened than those in the interior. There was no indication of necrotic breakdown of the cell structure, nor of apoptotic nuclear breakdown or vacuolar formation. A more rigorous test for cell death using Nile blue staining was thought unnecessary (Figure 4.3 A).

Experiment 4.04:

Injected cells maintained expression pCMVβ 24 hours after grafting:

In the final phase of control experiments, groups of 500 10T^{1/2} or COS-7 cells were injected with pCMVβ and then scraped off the dish as part of a larger square of 3000 cells, balled overnight and grafted into chick wings next day. The chicks were fixed and stained with X-Gal after 24 hours. All grafts had a number of blue cells showing that they could still express the introduced gene (Figure 4.3 D-H). The number of blue cells in the graft was typically ~20, whereas control injected squares which had been left on the dish showed up to 50 blue cells. This discrepancy may be due to increased cell death during removal from the dish and subsequent grafting. 5 balls of injected cells were stained without being grafted into the limb and showed reduced numbers of blue cells (10-20) so there is possibly cell death during removal from the dish.

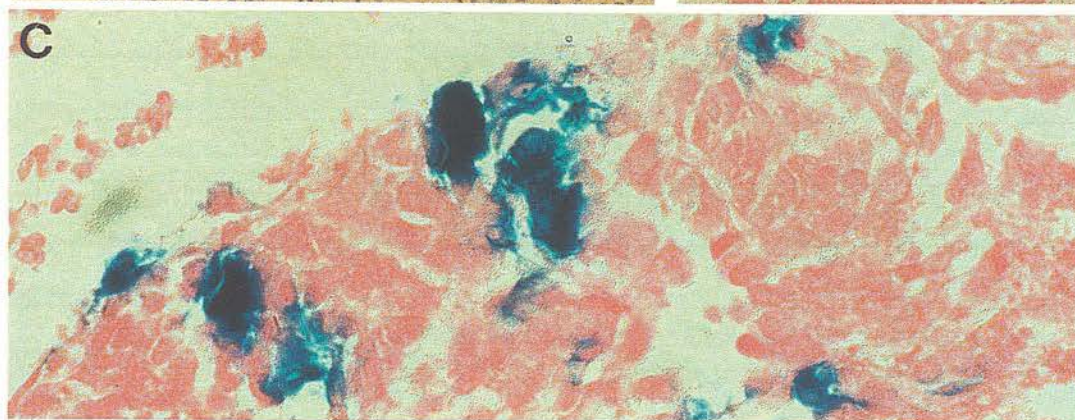
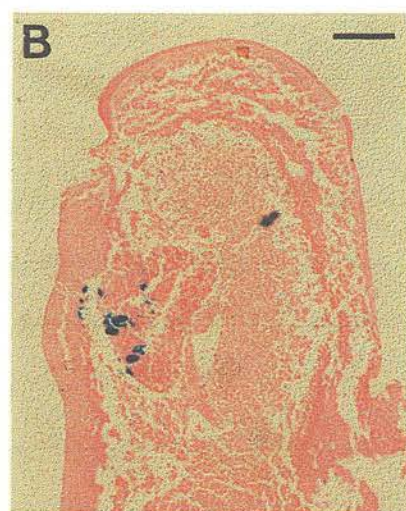
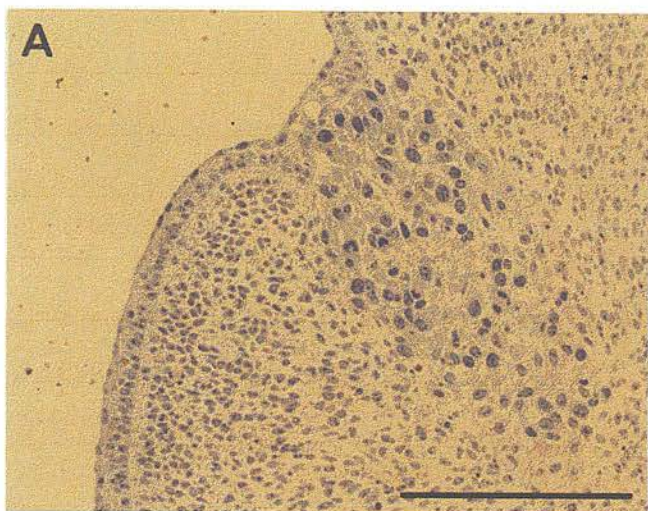
Some of the limbs containing positive blue grafts were wax-embedded and sectioned. This showed that blue cells could be located anywhere in the graft, and that the morphology of stained cells was identical to unstained ones (Figure 4.3 B,C).

Experiments 4.03 and 4.04

B - Section of grafted $10T\frac{1}{2}$ cells, injected with pCMV β , in the chicken limb. See Chapter 2 for comments on histology. C is an enlargement of B.

H - Grafted COS-7 cells, injected with pCMV β , in the chick limb

Scale bar represents 50µm



Injection of 10T^{1/2} cells with pH7lacT:

The control experiments had confirmed that, using 10T^{1/2} or COS-7 cells, it would be possible to introduce the *Msx1/lacZ* reporter constructs into chicken limbs to study the position-specific induction or repression of *lacZ*.

Primary limb mesenchyme cells would be the ideal cells to inject with pH7lacT and graft into the limb (since these would seem to be the most likely to be able to respond to endogenous limb signals). The problems of getting them to survive the time course of the experiment after microinjection seemed to preclude this. It would certainly be possible to attempt to alter the culture conditions, injection parameters etc. to attempt to obtain better survival for limb cells during microinjection; however, there was no evidence that the planned experiments absolutely needed to be carried out with transfected primary cells. All that is needed is cells which do not express the reporter in culture, but can do so in response to extrinsic signals in the limb. Published information on 10T^{1/2} cells (summarised below) suggested they may be a relevant cell line to use. It was decided therefore, in the first instance, to perform the experiments using 10T^{1/2} cells (COS-7 is not a relevant cell line for use in the limb); this would minimise cell death during the experiment, but would necessitate control experiments to study the expression and inducibility of endogenous *Msx1* in the cell line. An early result was the difference in behaviour between pH7lacT and pH7lacΔ3' in 10T^{1/2} cells (page 136), which was the spur to persist with the investigation of an interesting biological phenomenon in this cell line.

The rationale behind the choice of 10T^{1/2} cells:

CH310T^{1/2} cells are described on page 66. In terms of their growth responses and fibroblastic morphology they are very like Swiss and Balb/c3T3 cells. (Aaronson and Todaro, 1968; Reznikoff *et al.*, 1973). Fibroblast cells are fundamental in laying down positional information within the limb bud (Bryant and Gardiner, 1987) so the use of a fibroblastic cell line is appropriate.

10T^{1/2} cells can differentiate into cell types which are found in the limb:

The hypomethylating agent, 5-azacytidine, can induce 10T^{1/2}s to differentiate into limb-appropriate tissues in culture. Constantinides *et al.* (1977) showed that concentrations of 1-10μM 5-azacytidine induced 10T^{1/2} cells to undergo myogenic differentiation at low frequency in culture. They formed twitching multinucleated myotubules via a fusion of differentiating cells, 9-10 days after treatment.

Extending this work, Taylor and Jones (1979) showed that micromolar concentrations of 5-azacytidine could induce at least three new phenotypes in cultured $10T^{1/2}$ cells at low frequency, and also in 3T3 cells at lower frequency.

Culturing cells for 2-5 weeks after a 24 hour pulse of 5-azacytidine, they found that some cells had, in terms of a number of physical and physiological markers, differentiated into either striated muscle cells, adipocytes or chondrocytes. The adipocytic and myogenic activities, at least, were stably inherited in differentiated clones and hence represented an epigenetic modification of the pattern of gene expression in the cells. Davis *et al.* (1987) later showed that transfection with *MyoD* was sufficient to bring about the myogenic transformation.

Adipocytic differentiation can be caused by addition of hydrocortisone or ascorbic acid to the cells (Ibric *et al.*, 1988).

3T3 cells can respond to signals present in the limb:

There were further reasons for believing that $10T^{1/2}$ cells would be a relevant cell line to use. Trevino *et al.* (1992) showed that 3T3 cells, when grafted into the developing mouse limb bud, would adjust their proliferation rate to that of the surrounding limb mesenchyme cells (distal mesenchyme cells proliferate more rapidly than proximal cells). Importantly, this shows that there are non-autonomous signals in the limb bud controlling growth rate, and that 3T3 cells can read it and respond. Their response is limited - they do not differentiate into limb structures (Trevino *et al.*, 1989) - but the result suggests that $10T^{1/2}$ cells may also have the potential to respond to positional signals in the limb.

Summary:

3T3 cells were reported to die when grafted into chicken limbs (Tickle pers. comm.) although I did not attempt to confirm this myself. If true, it is a big difference between 3T3s and $10T^{1/2}$ s, since $10T^{1/2}$ cells certainly do not die (see results) which was a reason for using them! $10T^{1/2}$ cells, although an immortal cell line, have retained the ability to undergo some differentiation pathways under experimental manipulation and are responsive to external signals to bring about these changes. Given the *proviso* that differentiation *in vitro* took several days, and may have needed cell division and cell/cell interactions to bring it about, there is no inherent reason to believe that they would be unable to respond to limb-specific signals and undergo changes in gene expression.

Experiment 4.05

pH7lacT is not toxic, but is expressed at very low frequency in $10T^{1/2}$ cells:

Preliminary experiments were performed, injecting cultured $10T^{1/2}$ cells with pH7lacT at $\sim 0.5\mu\text{g}/\mu\text{l}$. Cells were then cultured for 24 hours, before fixing and staining with X-Gal. As controls, different cells on the same dish were injected with pCMV β ($\leq 0.1\mu\text{g}/\mu\text{l}$) and further cells were injected with a 1:1 mixture of the two plasmid solutions.

Initially, 4612 cells were injected with pH7lacT, of which 22 (0.48%) showed β -Gal activity after 24 hours.

In the same experiments, 1000 cells were injected with pCMV β , of which 81 ± 5 (8.1%) went blue. This is consistent with Experiment 4.02. With the 50:50 mixture of the two plasmids, 1100 cells were injected, of which 77 ± 5 (7%) went blue (Figure 4.5 G). This is comparable to the results obtained with pCMV β alone and shows that pH7lacT is not itself toxic.

Experiment 4.06

$10T^{1/2}$ cells, injected with pH7lacT, do not commonly induce expression of the construct in response to signals subjacent to the AER:

If the control of *lacZ* expression in pH7lacT reflected that of the endogenous *Msx1*, it would be expected that cultured, uninduced cells, would not be expressing the gene. Experiment 4.05 showed that this was very nearly the case. It remained possible, notwithstanding the few cells which expressed pH7lacT in culture, that control of expression in pH7lacT did reflect control of *Msx1* and, like *Msx1*, *lacZ* would be inducible by grafting underneath the AER.

In a long series of definitive grafting experiments (protocol shown in Figure 4.4), several (usually 5-6) blocks of 500 cells were injected with pH7lacT, one block of 500 cells injected with pCMV β and one block with a mixture of the two constructs. After allowing cells to recover for two hours in culture, all but one of the squares of pH7lacT-injected cells was cut from the dish as part of a 1.5mm square (~ 3000

cells) and balled up overnight as described previously. This left 3 squares of injected cells on the dish (one for each construct and one mixture) which were retained in culture.

Next morning, balls of injected $10T^{1/2}$ s were grafted either distally into the wing bud of a stage 20-21 chicken, or proximally into a stage 23-24, as described previously. Chickens were allowed to develop for a further 18 hours before fixing and staining with X-Gal. Injected cells on the dish were fixed and stained at the same time as the chicks.

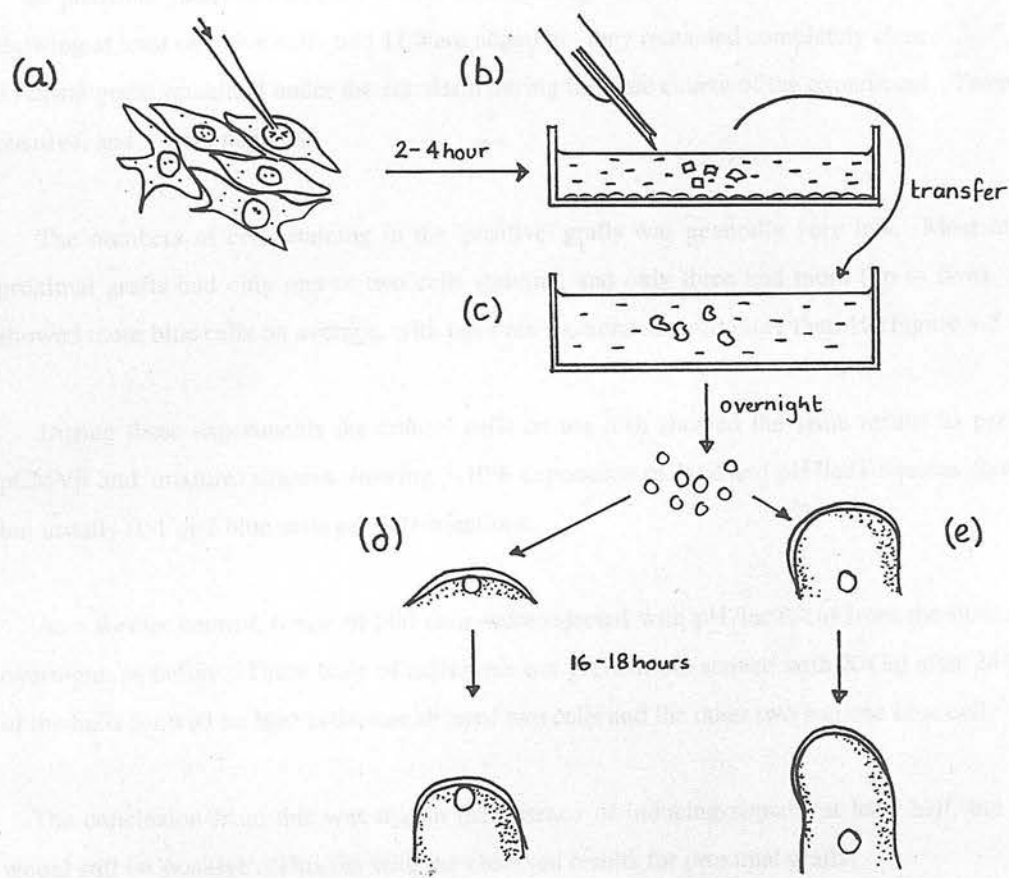


Figure 4.4:

Experiment 4.06 - Experimental procedure.

Four hours after microinjection (a), squares of injected cells were scraped from the dish (b) and transferred to non-tissue-culture petri-dishes in gassed incubators overnight. They were then grafted distally (d) or proximally (e) into chick wings, which were fixed and stained after 18 hours *in ovo*.

Grafts were classified as 'positive' or 'negative' for gene expression on the basis of the X-Gal. Because of the difficulty of counting exact numbers of blue cells in a graft embedded in an intact chicken, a graft was counted as positive if it showed just one or more distinct, intensely stained blue cell. Some grafts wholly went a pale blue colour if overstained and sectioning revealed that this was possibly due to bacterial infection. This was confusing at first, but there was in the end no difficulty in distinguishing these spurious blue colours from genuine blue cells, on the basis of intensity of staining and the speckled appearance of grafts which contained genuine blue cells.

22 proximal grafts survived to fixation and staining. Eleven were classified as positive on the basis of showing at least one blue cell, and 11 were negative - they remained completely clear.

33 distal grafts remained under the ectoderm during the time course of the experiment. Twenty-four were positive, and 9 were negative.

The numbers of cells staining in the 'positive' grafts was generally very low. Most of the positive proximal grafts had only one or two cells staining, and only three had more (up to five). Distal grafts showed more blue cells on average, with but even so, none showed more than 10 (Figure 4.5 A-F).

During these experiments the control cells on the dish showed the same results as previously, with pCMV β and 'mixture' squares showing 5-10% expression of *lacZ* and pH7lacT squares showing up to 4, but usually 0, 1 or 2 blue cells per 500 injections.

As a further control, 6 sets of 500 cells were injected with pH7lacT, cut from the dish and balled up overnight, as before. These balls of cells were not grafted, but stained with X-Gal after 24 hours. Three of the balls showed no blue cells, one showed two cells and the other two had one blue cell.

The conclusion from this was that in the absence of inducing signals, at least half, but not all grafts would still be 'positive'. This fits with the observed results for proximal grafts.

I performed a simple χ -squared test on the grafting results:

The null hypothesis is that there is no difference between the frequency of occurrence of the four classes of graft (proximal positive and negative, distal positive and negative). There is one degree of freedom.

Total number grafts = 55

In total, there were 35 positive grafts and 20 negative

Proximal grafts = 22; 11 positive, 11 negative

(Expected number, based on null hypothesis is 14 positive, 8 negative)

Distal grafts = 33; 24 positive, 9 negative

(Expected number, based on null hypothesis is 21 positive, 12 negative)

$$\begin{aligned}\text{therefore, } \Sigma((O-E)^2/E) &= 9/14 + 9/8 + 9/21 + 9/12 \\ &= 2.95 \quad (1 \text{ degree of freedom})\end{aligned}$$

This gives a probability of the null hypothesis being true of >10% i.e. there is no significant deviation from the null hypothesis.

There is however a deviation away from the null hypothesis, which is towards over-representation of positive distal grafts and negative proximal grafts. If it had been possible to count all cells and the number of positive cells in proximal and distal grafts had been taken into account then this difference would have been emphasised.

Conclusions:

There *is* a difference in the frequency of reporter gene expression between cells injected with pCMV β and those injected with pH7lacT, with cells expressing the former construct much more frequently than the latter. There was an indication that the frequency of lacT expression was increased by grafting it distally into the *Msx1* expressing region of the chick limb bud, but this induction was not proven to a statistically significant level. The deviation from the null (above) strongly suggests that the expression of pH7lacT in 10T $^{1/2}$ cells is modulated by grafting into the limb. It is clear however, that it is impracticable to use this technique as a means of finding the AER response element in truncated 5' promoter sequences, without significantly improving the number of cells which contain the reporters (so that any response in grafts is more obvious).

Injection of cells with pH7lacΔ3':

These experiments were performed to see if there was any difference in behaviour between pH7lacT and pH7lacΔ3'. Although they share the same sequence 5' of *lacZ*, pH7lacT has an extra 7kb of *Msx1* ORF and 3' sequence 3' of *lacZ*. It was thought possible that there may be regulatory sequences here which would affect expression of the construct.

Experiment 4.07

Injected 10T^{1/2} cells express pH7lacΔ3' at high frequency:

Control injections were performed as in the previous experiments. 10T^{1/2} cells in culture were injected with either a 0.1μg/μl solution of pCMVβ or pH7lacΔ3'. Twenty-four hours after injection, cells were fixed and stained with X-Gal.

2830 cells were injected with pH7lacΔ3', of which 243 (±10) went blue upon staining; this represents an 8.5% success rate. 45/500 blue cells (9%) were obtained from cells injected with pCMVβ.

There is no difference in the frequency of *lacZ* expression between cells injected with pCMVβ and pH7lacΔ3'. On the basis of this experiment and the fact that only 0.5% of cells express pH7lacT, it was concluded that unlike pH7lacT, expression of pH7lacΔ3' is possibly constitutive in cultured 10T^{1/2} cells.

Experiment 4.08

Frequency of pH7lacΔ3' expression is unaffected by grafting proximally or distally in the limb:

It remained possible that there were signals in the non-*Msx*-expressing regions of the limb which would turn pH7lacΔ3' off in cells which were grafted into these regions. Grafting experiments were therefore performed along the same lines as before. Cells were injected with pH7lacΔ3', balled up overnight, grafted either distally or proximally into chickens of the appropriate age the next day, and the chickens were fixed and stained after 18 hours.

Grafts were scored as being positive or negative on the same basis as before. 17 proximal grafts were performed. 15 of these were positive and 2 negative. 13 distal grafts were all positive. The number of cells staining in positive grafts was still small (up to 30 per graft), but this was more than in the equivalent grafts with pH7lacT and the same as that achieved with the control pCMVβ grafts (Fig. 4.5 H,I,J). The two negative grafts were probably results of accidental loss of injected cells during transfer between the

culture dish and the limb. There is no significant difference between the proximal and distal grafts and therefore no repressive signal was found to be present in proximal areas of the limb.

Conclusion:

pH7lacT therefore behaves differently to pH7lacΔ3':

It was concluded that there is a repressive element in the regions of pH7lacT which prevents constitutive expression of the construct. This element is not present in pH7lacΔ3' and is therefore presumably located in either the 3' region of the *Msx1* gene, or in the intron. The correct expression of pH7lacT in response to position dependant signals in the developing chick limb was not proven.

The difference in behaviour between pH7lacT and pH7lacΔ3' is an important result, and a first step in the functional dissection of the elements controlling expression of *Msx1*. The small numbers of blue cells obtained in all experiments, and the consequent difficulty in interpreting results, unfortunately makes this an unsatisfactory means of examining the *Msx1* promoter.

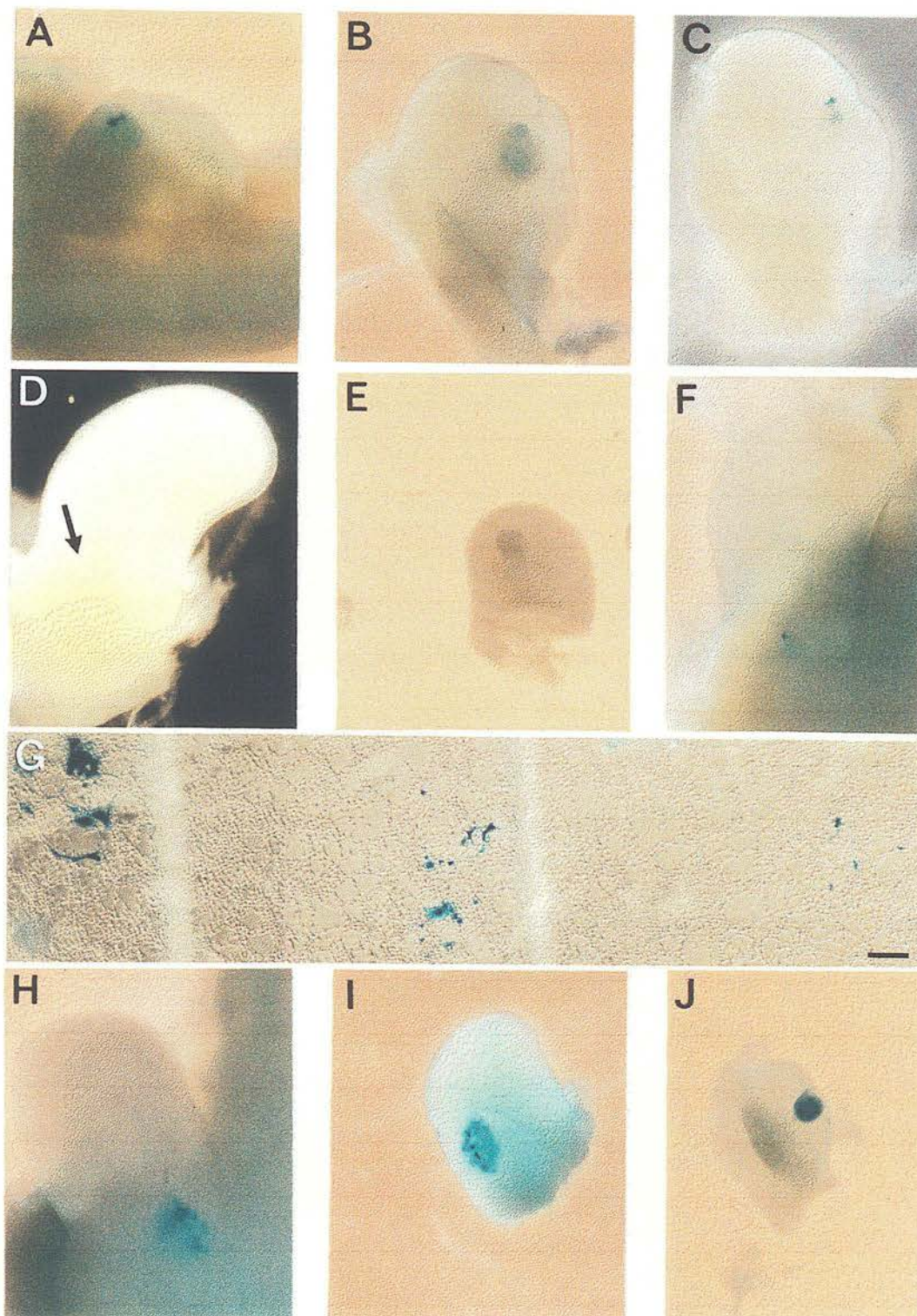


Figure 4.5:

A-F. $10T^{1/2}$ cells, injected with pH7lacT, grafted into chicken wings distally (A, B, C, E) or proximally (D, F). Very small numbers of cells stain after 18 hours *in ovo*

G. Equal numbers (200) of $10T^{1/2}$ cells injected with 100ng/μl pH7lacT (right), 50ng/μl pH7lacΔ3' (middle) or 50ng/μl pCMVβ (left). Fixed and stained after 24 hours. Scale bar = 100μm

H-J. $10T^{1/2}$ cells, injected with pH7lacΔ3', fixed and stained 18 hours after grafting into chicken

Part 2:

Introduction of DNA to cells by chemical transfection:

Alternative methods for the transient transfection of $10T^{1/2}$ cells:

Microinjection is a time-consuming and labour-intensive process which, during a day's work, produces enough material for 10 grafts at most, each of which probably only has ~50 successfully transfected cells. Transfecting cells chemically could produce unlimited amounts of grafting material, and furthermore, since transient transfection rates up to 80% have been reported for 3T3 cells it is feasible that each graft would have over 1000 transfected cells. This would make interpretation of the results much more straightforward.

3T3 cells are reported to die when grafted into chick limbs (Tickle pers. comm.). To investigate the possibility of using alternative transfection techniques on $10T^{1/2}$ cells I transfected 60mm cultures of these cells with pCMV β , using different chemical transfecting agents under different conditions of DNA concentration, cell confluence, transfection times and transfecting-agent concentration.

Cells were stained with X-Gal 24 hours after transfection to assay gene activity. Results were as follows:

Experiment 4.09

Transfectam:

% Cell confluence	μg pCMV β	μl Transfectam	% blue cells after staining
100	10	10	<1
100	10	20	<1
100	6	10	<1
100	4	10	<1
70	10	10	<1
50	10	10	<1
30	10	10	<1
30	10	20	<1
10	4	15	<1

There was no obvious difference, to a first approximation, between transfection efficiency under the various different conditions. Transfection efficiency was too low to be a useful replacement for injection. Cell death during transfection was high.

Experiment 4.10

Calcium Phosphate:

Transfection protocol was described in Chapter 2. Cells were transfected at 10, 30, 50, 70 and 100% confluence, using 5, 10 and 20 μg of pCMV β (i.e. 12 transfections).

As for Transfectam, changing the transfection conditions did little to change the efficiency of transfection as determined by X-Gal staining after 24 hours. All dishes showed very low (0.5-1%) occurrence of positive blue cells.

This experiment was repeated and still gave low transfection efficiencies which were inferior to those obtained by injection.

Experiment 4.11

DEAE Dextran:

Protocol as described in Chapter 2.

Cells were transfected at the recommended 70% confluence on 60mm dishes. In different transfections, 1, 2.5, 5, and 10 μ g of pCMV β were used, and 50, 150, 300, and 450 μ l of 0.1% DEAE Dextran in TBS (16 transfections). Cells were stained with X-Gal after 24 hours. The whole experiment was repeated three times.

Again transfection efficiency was low and varied little with changing conditions. On one occasion, transfection efficiency peaked at 1.8%, using 2.5 μ g pCMV β , 150 μ l DEAE Dextran (the recommended conditions), but in all other cases the efficiency was less than 1%, i.e. inferior to injection experiments.

Cell death was a problem during transfection procedure - DEAE Dextran is toxic; this effect was particularly obvious with higher concentrations of DEAE Dextran - cells would round up and detach from the surface of the dish whilst exposed to the Dextran.

10T $\frac{1}{2}$ cells produce a thicker extracellular matrix than many other cell lines and this may provide a barrier to DNA reaching the cell surface, which would explain their abnormally low rate of transfection. Reducing the confluence of the cells to 50%, which should have reduced this cell matrix, had little or no effect on the rate of DNA uptake.

It was concluded that unless a better method of transfecting 10T $\frac{1}{2}$ cells was found, the chemical transfection methods would not be an improvement on injection as a means of producing grafting material.

Use of stably transformed cell lines:

Previous experiments had been performed using transiently transfected cells. This was for several reasons.

Limb mesenchyme stops expressing *Msx1* and begins to chondrify within 72 hours of explantation from the limb environment. Although I have shown that *Msx1* can be reinitiated in non-expressing tissue, it is possible (although never tested) that the *Msx1* promoter becomes permanently silenced - i.e. it loses its ability to respond to inducing signals - once the cells start to overtly differentiate. In this respect, previous experiments have been a race against time, bearing in mind this possibility of promoter silencing. Since it takes 2-3 weeks to set up a cell line which has stably integrated ectopic DNA, this may be too long to ensure that promoter silencing does not occur in limb cells, even if it were possible to keep mesenchymal cells alive that long.

This argument does not necessarily apply to a cell line such as 10T^{1/2}. During the creation of a 10T^{1/2} cell line which has integrated the desired constructs, all that is required is to take cells in culture, transfect them, wait for two weeks in selection medium and then use them. There is no 'race against time' because (in contrast to primary cells) there are no differentiation processes occurring in the cells which might cause silencing of the introduced construct. The site of DNA integration might still affect the expression of the construct (by inserting near endogenous enhancers or within heterochromatin), but the spectre of *de novo* silencing, due to removing cells from their normal environment, does not appear.

So if pH7lacT or pH7lacΔ3' were introduced into the genome of 10T^{1/2} cells there is no reason why they should not retain the ability to express the construct. It is expected that some cells will integrate the gene into an area of the genome where it is subject to inappropriate position effects, or will disrupt the gene during integration, but by the same reasoning, it is expected that some cells will not. These cells may well express the reporter gene in a time and position-dependent manner which matches the activity of the endogenous *Msx1*. Using these cells to perform grafting experiment would obviously be beneficial, since a graft where 100% of cells have the ability to turn on the gene (compared to 10-15% of injected cells) would obviously give a much clearer indication of whether the promoter is activated.

One of the initial reasons for attempting the experiments described in this chapter was the speed with which it should be possible to analyse expression of the constructs in a large number of experiments. Production of a stably transformed cell line increases the time-span of a single experiment to at least three weeks, and negates this advantage. However, it became obvious that the analysis of the behaviour of the constructs in transiently transfected cells was handicapped by the low number of cells which were being

transfected. The production of cell lines in which all the cells had integrated the constructs seemed the best way of seeing what was really going on.

Experiment 4.12

Cell lines which have integrated the constructs show only very low frequency of expression:

Three cell lines were created, using calcium phosphate transfection methods to cotransfect pMCneo (constitutively expresses the neomycin resistance gene) with either pCMV β , pH7lacT or pH7lac Δ 3'

Cells were grown to 80% confluence in 100mm tissue culture dishes and then transfected using calcium phosphate with 0.5 μ g of pMCneo and 10 μ g of either pCMV β , pH7lacT or pH7lac Δ 3'. Selection in 800 μ g/ml G418 (which was sufficient to kill untransfected cells) was started after 16 hours. Cell death occurred and selection medium was replaced every 2 days for two weeks until the flasks had become confluent with neomycin resistant cells. Because cells transfected with calcium phosphate take up many copies of DNA, the 1:20 ratio of pMCneo to the other construct statistically ensures that any cell which integrates *neo*^r (usually as a concatemer) will also have integrated the other construct.

After 3 weeks in selection medium, by which time any transient (non-integrated) construct activity should have been lost, samples of the three cell lines, grown to confluence on 60mm dishes, were fixed and stained with X-Gal.

Surprisingly, none of the lines showed a high frequency of blue cells. The vast majority of cells remained unstained, indicating they were not producing β -galactosidase. About 0.5% of cells containing the pCMV β or pH7lac Δ 3' constructs went intensely blue, indicating *lacZ* was active.

The cell line containing pH7lacT showed weaker staining and in fewer cells (about 5x fewer cells than the two former constructs).

Staining was repeated on new samples of the cells, and gave the same results.

The experiment was repeated. Fresh 10T $\frac{1}{2}$ cells were taken, and calcium phosphate transfected with 0.2 μ g of pMCneo and either 2, 5 or 10 μ g of one of the three constructs (a total of 9 transfections). Selection for *neo*^r was carried out as before and staining with X-Gal performed after 3 weeks. The results were the same for all cell lines

The exact numbers of blue cells were not counted, since the exact percentage of expressing cells was not important (when it was too low to be of use in future experiments), and also because the differences

between the cells transfected with pH7lacT, pH7lacΔ3' and pCMVβ was small anyway. This differed widely from the expected result, based on expression of the injected constructs, which suggested that cells which had integrated pCMVβ would all express it, as would most or all cells that integrated pH7lacΔ3'. Few, if any, pH7lacT-integrated cells would be expected to express the gene (this is in fact what was seen).

Because expression of *lacZ* by the cells which have integrated pH7lacT was very infrequent, experiments were performed to see if frequency was increased by grafting cells underneath the AER.

Experiment 4.13

10T^{1/2} cells which have integrated the pH7lacT construct show no obvious induction of the gene in response to the AER:

3000-cell balls of 10TlacT cells were prepared as before. They were then grafted either distally into stage 20-21 chick wing buds, or proximally into stage 23-24 wings. After a further 18 hours, the chickens were fixed and stained with X-Gal.

Seven distal and five proximal grafts were performed. There was no clear difference between the proximal and distal grafts. All grafts showed very low numbers of blue cells, typically 0, 1 or 2. Intensity of staining in positive cells was usually at much lower than that seen by microinjection (Figure 4.6 B,C).

As a control, the dishes of cells from which the grafting material was cut were kept and stained at the same time as the grafted chickens. As previously, these showed expression in up to 0.1% of cells, which means that the average piece of graft tissue might be expected to have up to 2-3 expressing cells.

The experiment was repeated, performing a further 5 distal and 5 proximal grafts. All grafts showed 0-2 blue cells on staining.

Experiment 4.14

There is no induction of expression in $10T^{1/2}lac\Delta 3'$ cells in response to the AER:

Five distal and seven proximal grafts were performed using cells which had integrated pH7lac $\Delta 3'$. Again, few (up to ten) positive cells were seen on staining. All grafts had blue cells and there was no obvious difference between proximal and distal grafts, or between cells which had been left in the dish (Figure 4.6 D,E,F).

One proximal and one distal graft were performed using cells which had integrated pCMV β (Figure 4.6A). Both grafts showed about 5 blue cells.

Difference in gene expression between different $10T^{1/2}lacT$ cells is not a clonal effect:

There was no evidence from Experiment 4.13 that expression of *lacZ* could be induced in $10TlacT$ cells by grafting underneath the AER. The experiment was performed using a population of cells which had integrated the gene in different parts of the genome, and a large proportion of these cells may have been silenced by position effects - e.g. falling into heterochromatin or heavily methylated regions - or by gene disruption during integration. It was possible that a subset of cells in the graft were not subject to integration effects and were responding correctly to inducing signals in the limb, but that the effect was masked by the larger proportion of silenced cells. It was decided to derive clonal colonies of cells from this mixture of cells, and test them individually in grafts.

Richard Buckland took a confluent culture of these cells and obtained 23 clonally derived cell lines from single cells. He isolated DNA from 10 of the cell lines and using *lacZ*-specific primers for PCR analysis, showed that they all contained pH7lacT.

Experiment 4.15

Clonally derived cell lines do not show a response to the AER signal:

Initially, I performed one distal and one proximal graft into chicken wing buds using each of the cell lines (46 grafts). The behaviour of the cells fell into three categories:

- 15 lines showed a very few blue cells in both proximal and distal grafts;

- 1 cell lines showed blue cells in proximal grafts only

- 4 cell lines showed blue cells in distal grafts only.

- 3 lines showed no blue cells in either proximal or distal grafts

Surprisingly, grafts which did show blue cells after staining rarely showed more than 5 cells, and often much less, scattered throughout the graft. Hence, although the cells were clonally derived, they were not all responding in the same way in the limb environment (Fig. 4.6 G-L).

Of four promising cell lines which appeared only to express the introduced gene in distal regions of the limb (albeit not uniformly), one line died soon after the initial experiment. A further three proximal and three distal grafts were done for the three remaining lines (18 grafts). All three of these cell lines showed small numbers of blue cells now in all grafts, with no obvious difference between the numbers of blue cells in proximal and distal grafts. This was certainly not a uniform activation of the *Msx1* promoter in cells in response to limb signals.

There was still no tangible evidence of a response to the AER signal. No further experiments were performed with the cells at this time.

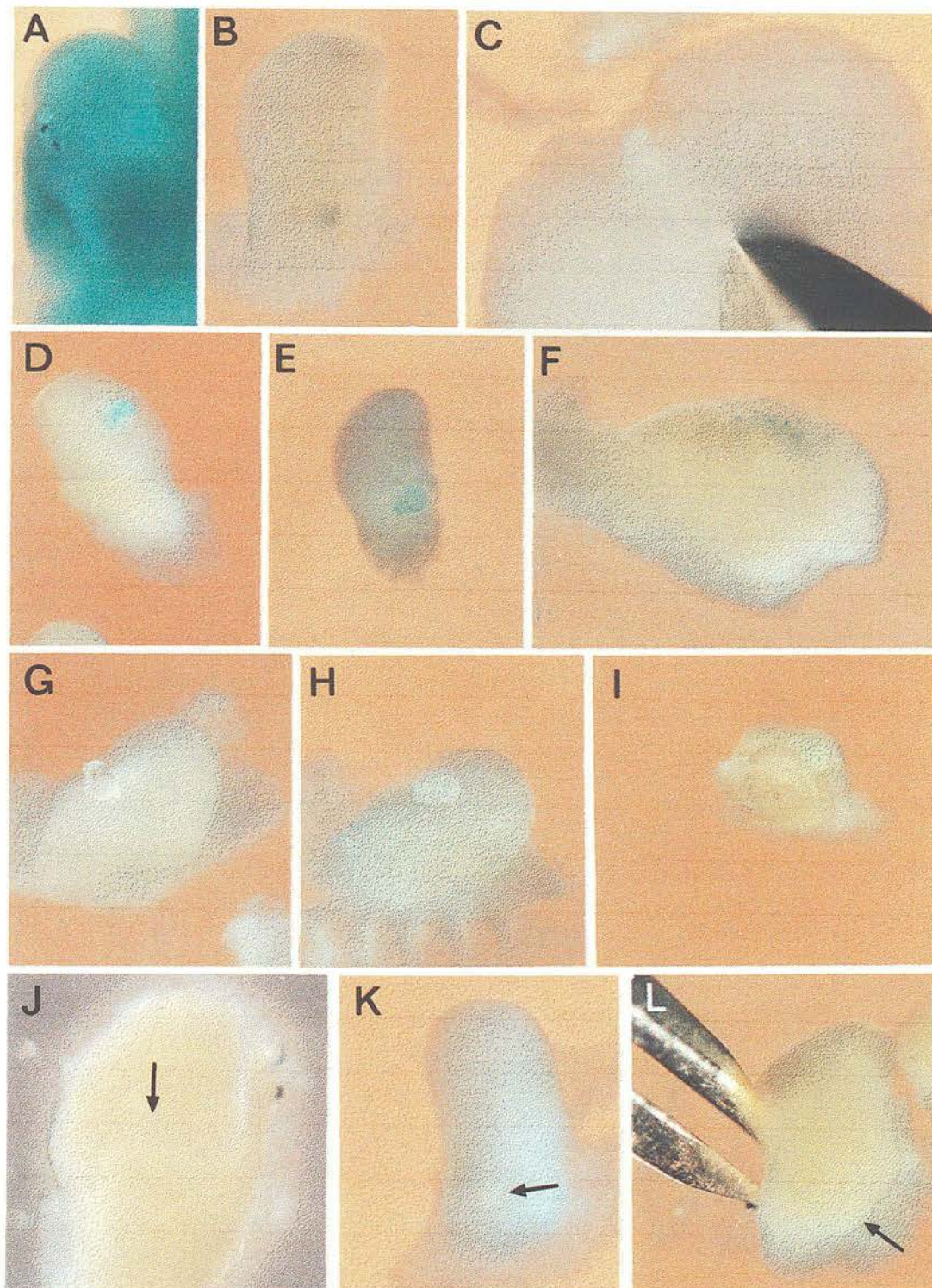


Figure 4.6:

Grafts of 10T^{1/2} cells which have stably integrated pCMVβ, pH7lacT or pH7lacΔ3', 18 hours after implantation into the chick limb

A. pCMVβ - this limb has gone blue in storage since staining.

B, C. Mixed population of cells which have integrated pH7lacT

D, E, F. Mixed population of cells which have integrated pH7lacΔ3'

G-L. Grafts of clonal cell lines which have integrated pH7lacT.

G,J - 10T31; H,K - 10T14; I,L - 10T26

Experiment 4.16

10T¹/₂ cells cannot express *Msx1* or *Msx2* in response to the AER:

The experiments described in this Chapter were overshadowed by the consideration that I was working with a cell line which had undergone epigenetic change leading to immortalisation. In a number of ways, these epigenetic changes could have resulted in cells now being unable to respond to the AER signal, such as by loss of the signal transduction pathways which lead to differentiation. It would obviously be meaningful to show that 10T¹/₂ cells could induce endogenous *Msx1* and *Msx2* in response to the AER signal, since this would imply that the lack of an AER response seen in the previous experiments was a function of the promoter and not of the cells.

Balls of 2000 10T¹/₂ cells were prepared as before and grafted distally under the AER of stage 20 chick wings. After 18 hours *in ovo*, the chickens were fixed, processed and sectioned for radioactive *in situ* hybridisation.

Probing with chicken *Msx1*, mouse *Msx1* and mouse *Msx2* on adjacent sections showed that 0/11 grafts, which were wholly or partly within the *Msx1* distal domain of the limb, showed any detectable *Msx1* or *Msx2* activity (Fig. 4.7).

I concluded that 10T¹/₂ cells are not capable of responding to the AER signal to induce *Msx* genes.

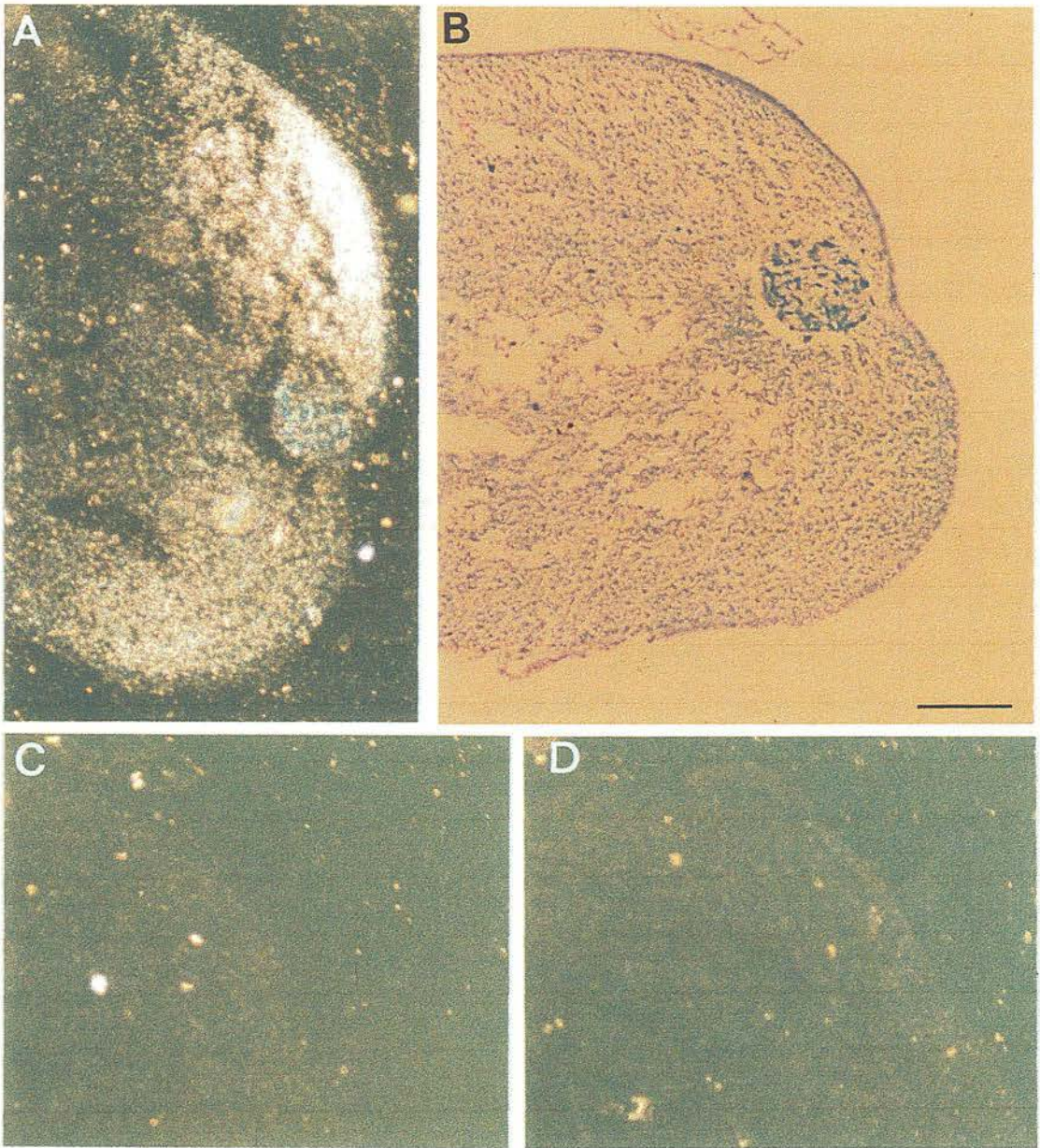


Figure 4.7

10T^{1/2} cells grafted subjacent to the chicken AER and cultured for 18 hours in ovo. B is bright-field. A, C, D are *in situ* hybridisations with chicken *Msx1* probe (A), mouse *Msx1* probe (C) and *Msx2* probe (D). This figure is representative of 11 grafts from 2 separate experiments, in both of which the mouse *Msx1* and *Msx2* probes were shown to label mouse mesenchyme.

Overview and conclusions:

There were obviously problems with the experiments which I was attempting. In a number of different experimental situations I had failed to demonstrate that the 4.7kb *MsxI* Nco5' promoter fragment was capable of driving *lacZ* expression in response to the AER signal.

I decided that I wanted to know why this was the case. Obviously, there was a problem with the cells (Experiment 4.16) but a failure to express the endogenous genes need not necessarily preclude expression of the introduced plasmid DNA. The fact that 10T^{1/2} cells do not express *Msx* genes when grafted under the AER neither invalidates the program of experiments nor necessarily means that 10T^{1/2}s were an unsuitable cell line.

The experiments would, for preference, be performed in limb mesenchyme cells, but I had been unable to maintain undifferentiated limb mesenchyme cells in culture after microinjection.

An unlimited supply of limb mesenchyme was available which contained the $\Delta 3'$ insert (*MsxI* Nco5' fragment in frame with *lacZ* and using SV40 processing and termination signals immediately 3' of the *lacZ* ORF) - lines of transgenic mice had been established in collaboration with CGR (Edinburgh) (1990). They were not however well characterised when I turned to them.

Since the behaviour of the *MsxI* Nco5' fragment was possibly an interesting biological phenomenon, I decided that the transgenic mice would provide another opportunity to investigate it. It was first necessary, however, to characterise the expression of the transgene, both in the limb and other areas of the body.

CHAPTER FIVE

Investigating the behaviour of the *Msx1* Nco5' promoter region in transgenic mice:

Introduction:

As described in Chapter 1, a number of transgenic mouse lines had been generated, which contained the $\Delta 3'$ construct - 4.7kb of *Msx1* immediate proximal 5' sequence, in frame with the *lacZ* ORF. Preliminary observation had suggested (R. Hill pers. comm.) that although they showed some aspects of an *Msx*-like expression pattern, each of the lines differed from the others in ways which were probably attributable to position-related effects on ectopic genes which have passed through the germline. However, these transgenic animals were potentially valuable, as a source of limb mesenchyme with which to investigate behaviour of the $\Delta 3'$ construct in grafting experiments mirroring those described in Chapter Four.

Three lines are currently maintained, $\Delta H5$, $\Delta H6$ and $\Delta H1$. $\Delta H5$ was reportedly the one which showed the most *Msx*-like expression pattern, so I investigated embryonic expression of the transgene (by X-Gal analysis) in these mice. The analysis was not intended to be comprehensive. It was intended firstly to compare the expression of the transgene in the limb with that of the endogenous *Msx* genes, secondly to investigate whether the transgene was expressed in an *Msx*-like pattern elsewhere in the embryo. I wanted to check whether expression of the transgene was a function of the promoter, or a position effect, and to see which aspects of *Msx1* expression were reflected by the transgene. In order to plan a series of experiments using transgenic tissue, it was necessary to know that the behaviour of the transgene in experimental situations would be a function of its promoter.

Results:

The 4.7kb of *Msx1* 5' sequence drives *lacZ* expression in a consistent pattern which approximates to elements of endogenous *Msx1* expression:

Expression of the transgene was examined by X-Gal staining in mouse embryos from E8 (10 somite stages) to E17. At least 10 embryos were examined for each stage.

5.01

Transgene expression in the limbs of $\Delta H5$ mice - a comparison with the expression of endogenous *Msx1* and *Msx2*:

The transgene is expressed throughout the lateral plate mesoderm, caudally from the anterior of the fore-limb field from E8. The medial boundary of expression closely matches that of *Msx1* (pers.obs.; Davidson, pers.comm.) It is also expressed until E10 in the posterior-ventral mesoderm of the 'tail bud' (Figures 5.1A; 5.4D) - a region of proliferating cells with the same developmental potential as the primitive streak. This probably means that the transgene is expressed in the primitive streak during gastrulation, like *Msx1*.

Expression in flank mesoderm is downregulated from ~E9.5; the transgene remains expressed throughout the mesenchyme of the nascent fore- and hind-limb buds. Expression pattern in the fore- and hind-limbs is similar at equivalent developmental stages. The following detailed description is for the fore-limb.

Early expression - W1 to W3:

Transgene expression approximates well to endogenous *Msx1* - it is expressed in nearly all cells of the mesenchyme of the budding limb (Figure 5.1; D,I). The anteriorly biased arc of gene expression (W1, W2) never occurs, which may be a consequence of perdurance of β -Gal. This would lead to β -Gal being detected in a more extensive domain than *Msx1* message.

Unlike *Msx* genes, there is no transgene expression in the limb ectoderm prior to AER formation (Figures 5.1I; 5.2B)

The AER/Progress Zone relationship:

The transgene is expressed strongly throughout the AER upon its induction (W3). Expression is maintained through stages W4 to W8, and is last detected in the ectoderm at the distal tips of the separating digits at W9 (E13). There is no other ectodermal expression.

Intense, prolonged, expression in the AER is more similar to *Msx2* than *Msx1*.

From W4 onwards, the transgene is downregulated in the most distal mesenchyme. β -Gal is henceforth undetectable in most of the mesenchyme subjacent to the AER. Expression is maintained in cells at the anterior margin of the limb, proximal to the digit plate (Figure 5.1E,F,G,J,K). There is also a small domain of *lacZ*-expressing cells subjacent to the posterior margin of the limb.

There is therefore no transgene expression in the distal mesenchyme which would ensue from response to an AER signal. The AER is induced after W3. At this stage transgene expression is strong in the subjacent mesenchyme, becoming weaker at W4 and absent at W5. This is consistent with a failure of maintenance of transgene expression by the AER, and a subsequent fading away of β -Gal within 12 hours.

W5 (E11.5) onwards:

At W6, positive mesodermal cells are restricted to a discrete block within the anterior of the zeugopod, (and a few cells in the postero-ventral mesenchyme of the hand plate). At W7 and W8, the anterior block of blue cells is recognisable as developing muscle. At days 14.5 and 15.5 (W10 onwards), transgene expression becomes limited to 3 of the pectoral muscles and two of the anterior muscles of the forearm (one strongly and one weakly) (Figure 5.1G,H,L,M). Expression in these muscles is maintained until W13. (Expression in the posterior ventral handplate is lost after W7).

Expression in the hind-limb differs from that of the fore-limbs only in the exact shape and position of the anterior and posterior 'muscle forming' domains.

Interdigital expression:

β -Gal is undetectable interdigitally, either in the zones of cell death, the perichondrium or in the soft tissue surrounding the chondrifications. This is in contrast to *Msx1* and *Msx2*. Thus, after W9, the only expression in the limb was in the muscles of the forearm, as described above.

Conclusions:

Transgene expression in the limb shows some features of *Msx1* expression - in the AER and throughout the mesenchyme of the early limb bud. Expression fades prematurely in the Progress Zone, however, is not present in the ectoderm prior to AER formation and is not reinitiated in the autopod to coincide with cell death.

Cells expressing the transgene in the limb mesenchyme after 11.5d are largely, and probably wholly, a population of myogenic cells.

As stated in Chapter Three, *Msx1*, but not *Msx2*, is expressed in the ventrolateral cells of the dermamyotome which will migrate away to form the limb musculature. In transgenic mice, the newly-formed 'epithelial ball' somites are negative for β -Gal activity (Figures 5.1B; 5.2A) transgene expression initiates *de novo* in the ventrolateral region of the cells that become the dermamyotome and hence the dermatome. Blue cells then bud off between E9 and E10 into the intermediate mesoderm between the somite and the limb (Figure 5.2B). I suggest that the domains of blue cells in the transgenic limb from W4 onwards are a subset of these myogenic cells which have retained transgene expression, but which are not distinguishable until the rest of the limb goes clear. At W4, they show a patchy distribution, blue cells being interspersed between clear cells (Figure 5.2C). Paralleling the described description of myocyte behaviour in the limb, these blue cells aggregate into a discrete block (Figure 5.2D) which bifurcates during development so that expression becomes restricted to a few discrete muscles (Figure 5.2E,F).

The anterior and posterior regions of transgene expression, in domains of differentiating myocytes which only partly overlap with the expression of *Msx1*, is probably an 'ectopic' expression pattern. It may be under a different regime of control from the endogenous gene at this stage.

Expression of the transgene in the limbs of other lines of ΔH mice:

The expression of $\Delta H5$ was compared with expression in $\Delta H6$ and $\Delta H1$ mice, and in preserved specimens of several other lines.

Anomalous limb expression of the lac $\Delta 3'$ insert was not a peculiarity of $\Delta H5$. Of the other lines, $\Delta H8$, $\Delta H3$, $\Delta H30$, $\Delta H37$, $\Delta H10$, $\Delta H1$ and $\Delta H6$ show similar patterns, with small differences in the number and intensity of myogenic cells which retained transgene expression after E11 (Figure 5.4A,E,G,H). Only $\Delta H29$ was very different, showing weak staining in only a few cells of the limb.

A number of transgenic embryos produced by Alasdair Mackenzie in 1994, using the lac $\Delta 3'$ construct, showed the same limb expression patterns, again with variable strength of myogenic staining.

5.02

Other aspects of transgene expression in $\Delta H5$ mice:

The transgene's promoter drives an expression pattern which is similar, but not identical, to *Msx1* in other areas of the body.

Central nervous system:

I examined embryos from the E8, 12 somite, stage onwards. At this point, neural crest migration is underway at the level of fore-, mid- and hindbrains.

The transgene is expressed at the dorsal margins of the head folds and the neural tube, both before and after closure, in an *Msx*-like domain (Figures 5.1A,B; 5.4D). Expression is continuous along the neural tube caudal to the hind-brain until E10.5, after which it is only detectable in a scattered cells at the dorsal suture of the tube.

In the brain, there is weak expression in the rhombomeres from E8.5-E10. This is rather uniform and not restricted to specific rhombomeres (Figure 5.1A).

Expression is just detectable in the dorsal folds of the presumptive forebrain before closure, but only in superficial cells at the suture the prosencephalon after closure.

Staining is strongest in the dorsal folds of the mid-brain, from E8 onward (Figure 5.1A,B). After closure of the folds, expression remains at the midline of the brain (Figure 5.1C) and in the pineal gland until E14 (Figure 5.3E). Some epithelial cells laterally in the superficial layer of the mesencephalon also show β -Gal activity (Figure 5.3D).

Neural crest:

All neural crest cells caudal to the hindbrain express the transgene as they delaminate from the roof of the neural tube (Figure 5.3A). Expression is very transitory (like *Msx1*). The neural crest-derived structures of the trunk are not blue. Some staining in the dorsal root ganglia (best seen in the $\Delta H6$ mouse, Figure 5.4A), (products of the last wave of neural crest migration (Serbedzija, 1990)), is possibly residual from the migrating crest. (This expression has also been seen in *Msx2/lacZ* transgenics (Liu *et al.*, 1994)).

Cranially, expression is seen in scattered migrating neural crest cells at the level of the mesencephalon from E9.5-E11.5 (There is expression also in the overlying head epithelium and superficially in the brain at this time) (Figures 5.1B,C; 5.3B)

In contrast to *Msx1*, there is no expression of the transgene in forebrain-derived neural crest.

Neural crest streaming from rhombomeres 2, 4 and 6 between E8 and E9 does not show β -Gal activity (Figure 5.1A,B). This is in contrast to the situations with the *Msx2/lacZ* transgenics and endogenous *Msx1*. The trigeminal ganglion, a 1st arch neural crest derivative (Serbedzija, 1990), is weakly blue (visible in Figure 5.1B), possibly indicating some activity in the migrating crest.

As a result of the very limited β -Gal activity of the migrating neural crest, in contrast to *Msx1* expression, most of the dorsal neural crest derived mesenchyme in the head and trunk is negative for transgene activity.

The face:

The mesenchyme of the first and second branchial arches does not initially express the transgene at E9 (probably because of deficient expression in the migrating neural crest), but from E9.5, β -Gal activity is detectable (Figure 5.3C), becoming strong by E10.5 (Figure 5.1C). Expression in the hyoid arch is weak and transient, disappearing by E12.5.

Distal mesenchyme of the mandibular process shows graded expression, strongest laterally and distally, which reflects the reported expression of the endogenous gene (Figure 5.3D). Expression is weaker, but detectable in the mandibular epithelium (Figure 5.3D). Mandibular staining is observed until E16 in the masseter muscle and connective tissue laterally in the jaw (Figure 5.3G).

There is no expression in the mesenchyme of the maxillary or fronto-nasal processes (in contrast to *Msx1*) although the epithelium of the maxilla is positive. There is strong expression in the thickened epithelium of the lateral nasal process from E9 to E12, and henceforth at the apertures of the nostrils and in the internal sinusal epithelia (Figure 5.3H).

There is no expression in the teeth (Figure 5.3G) in contrast to endogenous *Msx* genes. The teeth of transgenic animals appeared to be developmentally retarded when compared to wild-type littermates.

Otic vesicle:

β -Gal activity is detected in the dorsal epithelium of the involuting and involuted otic vesicle between E9 and E11, a pattern which is at least partly reflected by *Msx1*.

Eyes:

Transgene expression is detected at the dorsal neural retinal layer of the optic cup at E11 and then weakly around the lip of the optic cup. It is now clear (Holme pers. comm.) that this represents an *Msx1* expression domain. At least some of the blue cells are destined to become ciliary body.

Heart:

$\Delta H5$ mice show early expression in the endothelium of the heart from E8.5 like *Msx1*. In contrast to *Msx* genes, no expression was detected in the endocardial cushions, semi-lunar valves or myocardium.

Somites:

The expression in dermamyotome and migrating myogenic cells at the level of the limbs has already been mentioned. Newly formed 'epithelial ball' somites do not show β -Gal activity. The ventrolateral quarter of all somites from the anterior of the fore-limb to the posterior of the hind-limb then starts to express weakly and the blue becomes restricted to the cells at the ventral lip of the dermamyotome. This process can be seen by looking at somites along the a-p axis of the embryo (Figure 5.1B).

Ventrolateral dermamyotome between the fore- and hind-limbs also expresses the transgene between E9 and E11.5 (Figure 5.1C). These cells will migrate away and form the muscles of the body wall, but not before transgene expression is lost.

It is noteworthy that only the somites of the limb field show blue ventral dermatome. The 4 most anterior somites, whose ventrolateral cells will migrate to form the muscles of the tongue (Wachtler and Christ, 1992) do not show this β -Gal activity. The transgene therefore provides molecular evidence for genetic specification of the limb field.

It is not known whether *Msx1* is expressed in all somites of the limb field, or whether precise timing of expression coincides with that of the transgene.

Gut:

There was β -Gal activity in a restricted domain within the ventral endoderm of the gut at E9 and E10.

Urogenital system:

Expression in the ventral posterior tail-bud at E9 and E10 included mesoderm of the developing urogenital ridge. At E11.5 there is expression in the epithelium and mesoderm around the cloacal opening which was localised at E12 and E13 to the mesoderm of the genital eminence and swellings.

It is not clear how closely this represents the expression of *Msx1*.

Most aspects of transgene expression are conserved between lines:

Comparison of $\Delta H5$ with $\Delta H1$ and $\Delta H6$, as well as Alasdair's transient transgenics and preserved specimens of other ΔH lines, confirmed that most of the above aspects of expression were not peculiarities of the $\Delta H5$ line, and that they probably represented the genuine capabilities of the 4.7kb of promoter sequence (Figure 5.4). Most lines express in the same places at the same times, although there were quantitative differences in expression between different lines in different structures. $\Delta H6$, for example, expresses strongly in neural structures, such as the dorsal root and trigeminal ganglia, but is only weakly expressed in the lateral plate and neural crest derived mesenchyme - in contrast to $\Delta H5$. These quantitative differences indicate that the promoter is very specific with regard to expression sites, but is sensitive to variation in absolute levels of expression at the different sites - this sensitivity probably reflecting integration site.

Main differences between lines are:

$\Delta H1$ expresses the transgene in a peculiar pattern amongst the superficial neurons on the hind- and mid-brain; there was no expression in the otic vesicle. In the E8.5 embryo, the transgene shows transient upregulation in rhombomere 4 - in direct contrast to expression of the endogenous *Msx* genes. In later embryos there was expression in the epithelium of the developing nipples (a feature shared with $\Delta H30$). $\Delta H1$ was also expressed in the lens at E10-12 (as was $\Delta H29$).

Many lines show expression at the dorsal lip of the dermamyotome of all somites from E9 to E11.5, from which cells migrate round the inner surface of the somite to form the myotome. This was particularly strong in $\Delta H6$ (Figure 5.4B), $\Delta H8$ and $\Delta H10$ but was at the limit of detectability in many lines, including $\Delta H5$.

$\Delta H6$ shows expression in small numbers of cells at the dorsal root and more ventrally in the neural tube (Figure 5.4C).

Conclusions:

This analysis, albeit not exhaustive, indicated the following:

- (i) That the expression of the transgene shows some aspects of expected *Msx1*-like expression, but is not a perfect representation of the endogenous gene.
- (ii) Whilst there are differences between transgenic expression in different lines, these are largely quantitative, and there are enough similarities to be confident that the patterns seen are a genuine

representation of the capabilities of the promoter - they are not due solely to position effects. However, some differences between lines probably indicate that the promoter is at least partly susceptible to influences from surrounding enhancers at the site of integration.

When looking at patterns of *lacZ* expression, one must consider the possibility of perdurance of β -gal protein. I have already suggested that this may explain staining in the ganglia, although there is no evidence that there is no *de novo* transcription in these structures. On the whole, the pattern of β -Gal activity is quite dynamic, and there are specific cases e.g. in the flank and limb mesenchyme, where expression drops very sharply in a short space of time, which suggests that perdurance is a matter of hours, not days.

The following table compares expression of the transgene with expression of *Msx1*. In the transgene column, ‘++’ indicates that the transgene follows very closely the pattern of *Msx1*; ‘+’ indicates that there are differences in timing or exact pattern of expression. ‘-’ indicates a site of *Msx* expression where the transgene is not expressed.

Midbrain/Neural crest	
Trunk	
Forebrain	
Midbrain	Only the lacZ gene defines this
Limbic axis	Not seen
Neuro-ectoderm (ventral midbrain)	
Mesoderm	
Chondro mesoderm (E12-E17)	Neuroectoderm present
Pharyngeal Gland (E11-E13)	Msx1 is expressed, but not lacZ
Whorled (E14-E16)	
Not yet defined	

<i>Msx1</i>	Transgene	Comments
<u>Primitive streak</u>	?	
<u>Mesoderm of ventral tail ridge</u>	+	Transgene more extensive
<u>Early lateral plate</u>	++	
<u>Dorsal neural tube</u>		
Trunk	++	
Hindbrain (i) r3+r5	-	
(ii) later, uniform	+	Very weak
Midbrain E8-E10	++	
Forebrain	+	Weak
E11.5 dorsal diencephalon	-	
<u>Migrating neural crest</u>		
Trunk	++	
Forebrain	-	
Midbrain	+	Only the last wave retains blue
Hindbrain	-	Not seen
<u>Neural crest-derived dorsal head mesenchyme</u>	-	
<u>Meninges</u>	?	
<u>Choroid plexus E 12-E17</u>	++	Mackenzie pers. comm.
<u>Pineal Gland E11-E14</u>	?+	<i>Msx1</i> is expressed, but undefined
<u>Whisker follicles</u>	-	
<u>Rathkes pouch</u>	-	

Facial Processes E9-E11

Mandibular mesenchyme	++	
Mandibular epithelium	++	
Maxillary mesenchyme	-	
Maxillary epithelium	++	
Hyoid mesenchyme	+	Weak
Hyoid epithelium	-	Not seen
Fronto nasal mesenchyme	-	
Fronto nasal epithelium	-	
Lateral nasal mesenchyme	-	
Lateral nasal epithelium	++	

Dental mesenchyme

-

Branchial clefts

++

Jacobson's Organ

-

Somites

++

?Shoulder musculature

+

More prolonged

Eye

Ciliary body

+

Lens

+

Msx2-like (few lines)

Otic vesicle

++

Heart

Cardiac endothelium

++

Endocardial cushions

-

Semilunar valves

-

<u>Limb mesenchyme</u>		
Early	++	
After AER formation	-	
Interdigital	-	
Perichondrium	-	
<u>Limb ectoderm</u>		
Pre-AER	-	
AER	++	<i>Msx2</i> -like
<u>Urinogenital system</u>		
Genital eminence	+	<i>Msx1</i> data incomplete
Genital tubercle	?-	
<u>Hair follicles</u>		
	-	
<u>Nipples</u>		
	+	$\Delta H1$, $\Delta H30$ only

Sites of transgene expression where *Msx1* is not found:

- Dorsal root ganglia
- Trigeminal ganglion
- Muscles of limb after E11
- Roof of mesencephalon after E11

Because the promoter was driving a dynamic pattern of *lacZ* expression during development in a predictable, *Msx*-related, pattern, I decided that it would be possible to investigate the behaviour of the promoter in experimental situations. These experiments were to be aimed at investigating the signals to which the transgenic promoter could respond and seeing how and when this differed from the behaviour of the endogenous *Msx1*.

These experiments will be described in the next chapter.

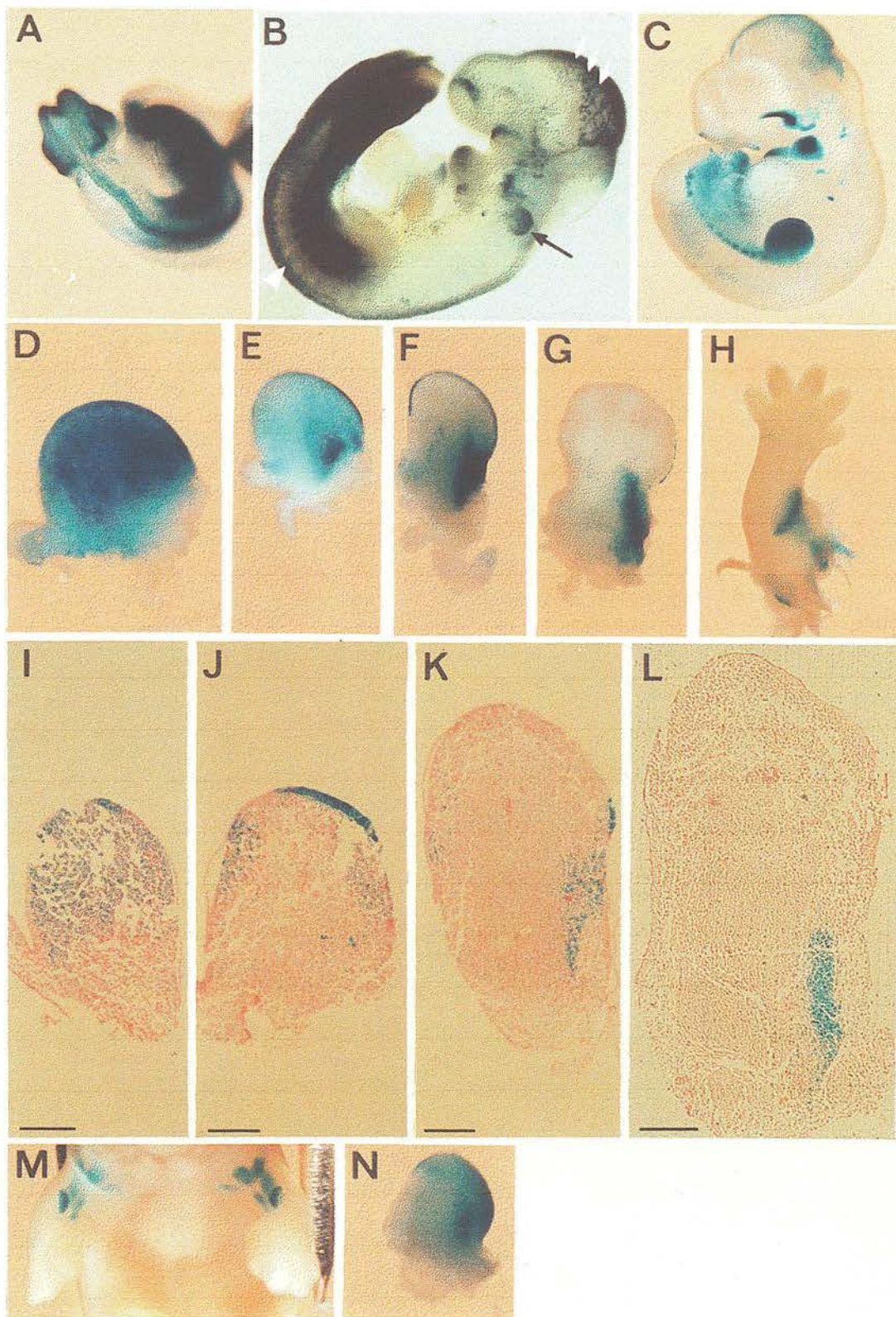


Figure 5.1: ΔH5 limb development

A, B, C - ΔH5 whole embryos. A=E8.5; B=E9.5 (W1); C=E10.5 (W3). The black arrow in B points to the otic vesicle. The three white arrows point at migrating mesencephalic neural crest. The white arrowhead shows the newly-formed somites, initially negative for β -Gal activity, with *lacZ* expression being initiated *de novo* in older somites.

D-L Whole limbs (D-H) or longitudinal sections (I-L) of ΔH5 limbs. Anterior is to the right. Scale bar represents 50 μ m. Only fore-limbs are shown.

D,I=stage W3 (E10.5); E,J=W4/5 (E11); F,K=W5/6 (E11.5); G,L=W8 (E13); H=W12 (E14.5).

M is a frontal view of an E13.5 embryo, showing blue muscle blocks in the anterior of the limb.

N is an E11.5 hind-limb.

Figure 5.2

Migration and development of β -gal positive cells from the somites at the level of the limb (see text)

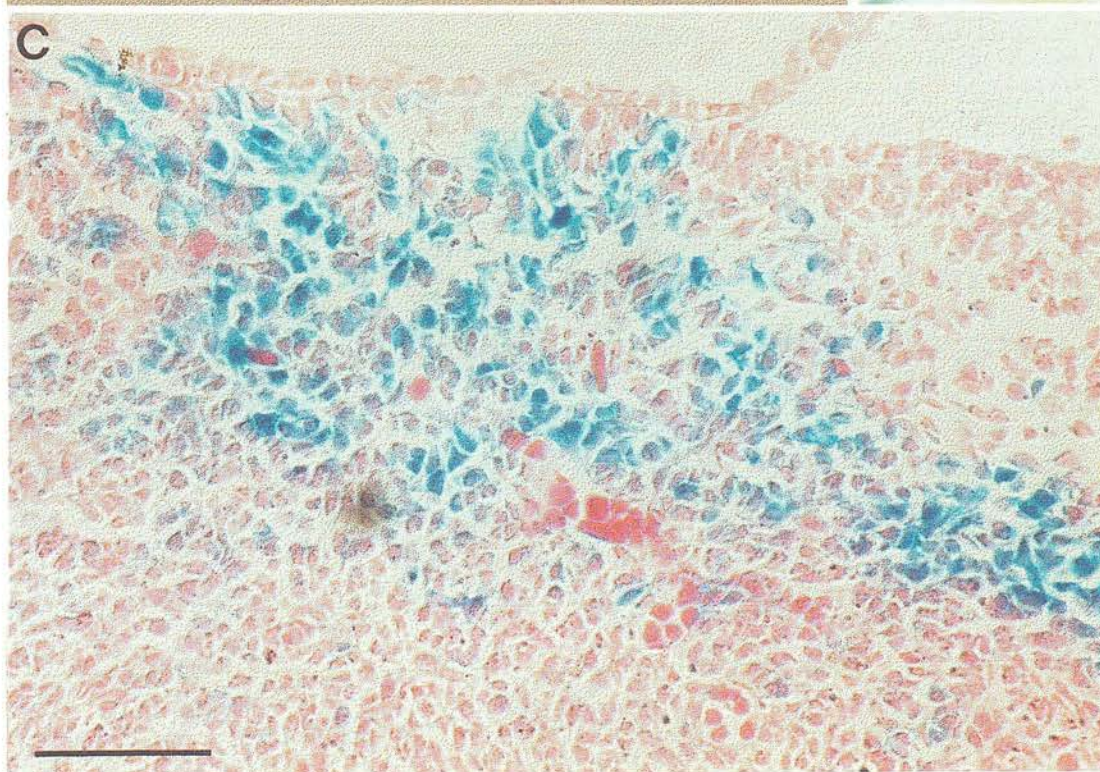
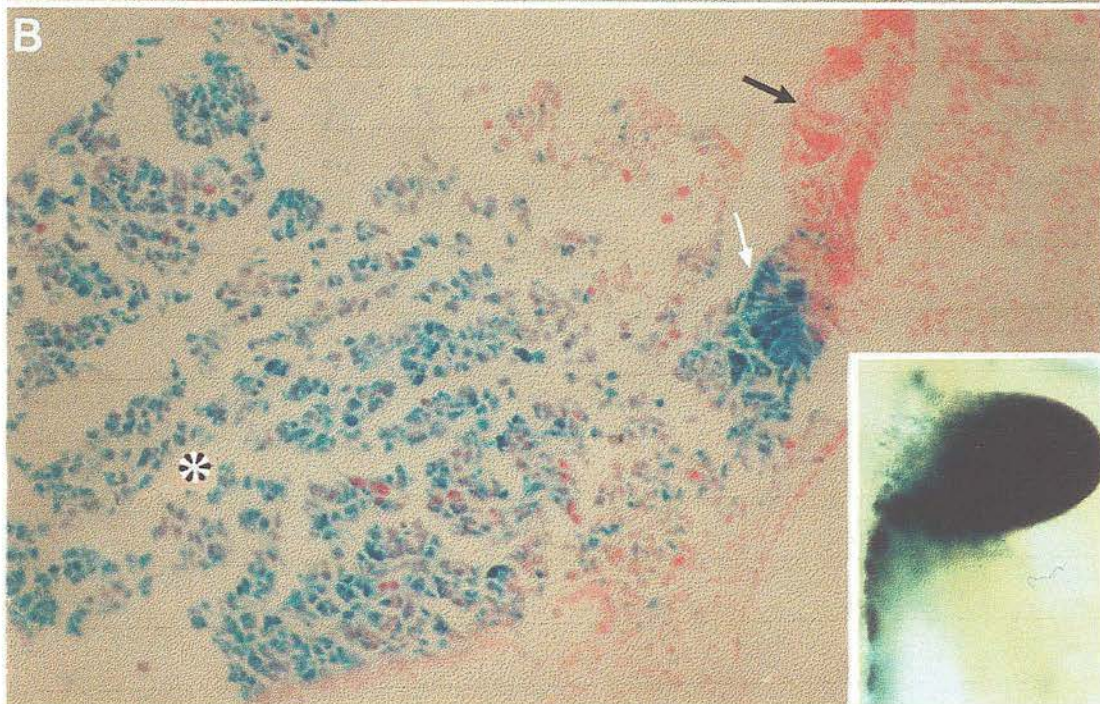
A. Transverse section of an E8.5 embryo. The newly-formed somites are negative.

B. E10 fore-limb (W3). The black arrow shows the dermatome, with the white arrow showing the cells at the ventrolateral edge, which are blue. From here, cells can be seen budding off (also see insert). They migrate to, and are lost in, the blue limb mesenchyme (asterisk).

C, D. Longitudinal sections of fore-limbs. C=E11.5 - blue cells accumulating in the anterior of the fore-limb. This is an enlargement of Figure 5.1K. D-E12.5 - a block of myoblasts in the limb.

E, F. Transverse section of a E14.5 fore-limb. Muscle blocks are visible, with blue myotubes in the anterior blocks. F is an enlargement of E.

Scale bars - A, B=10 μ m, C-F=50 μ m



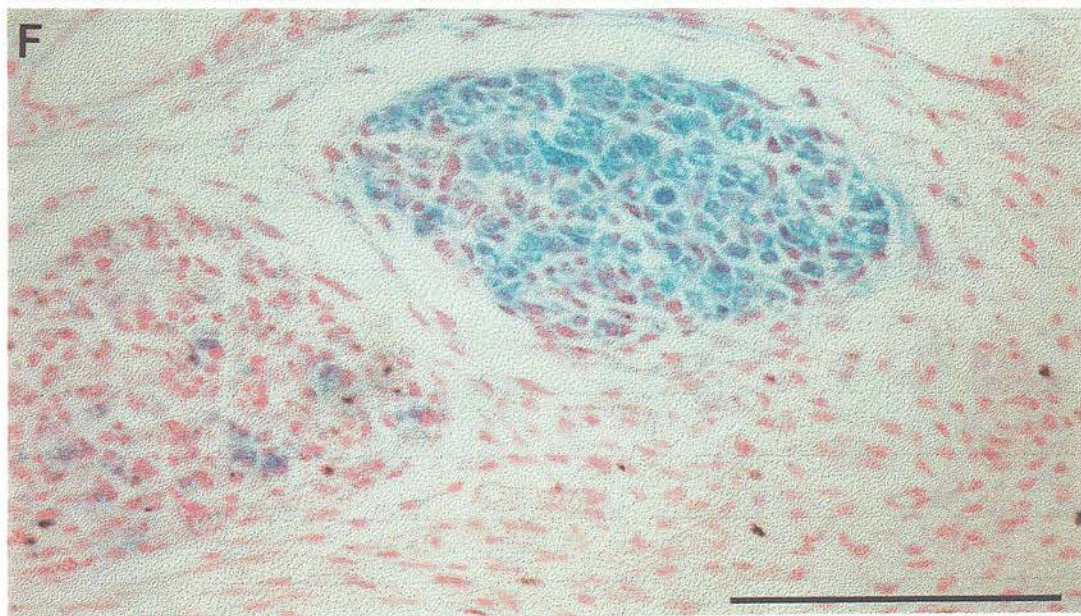
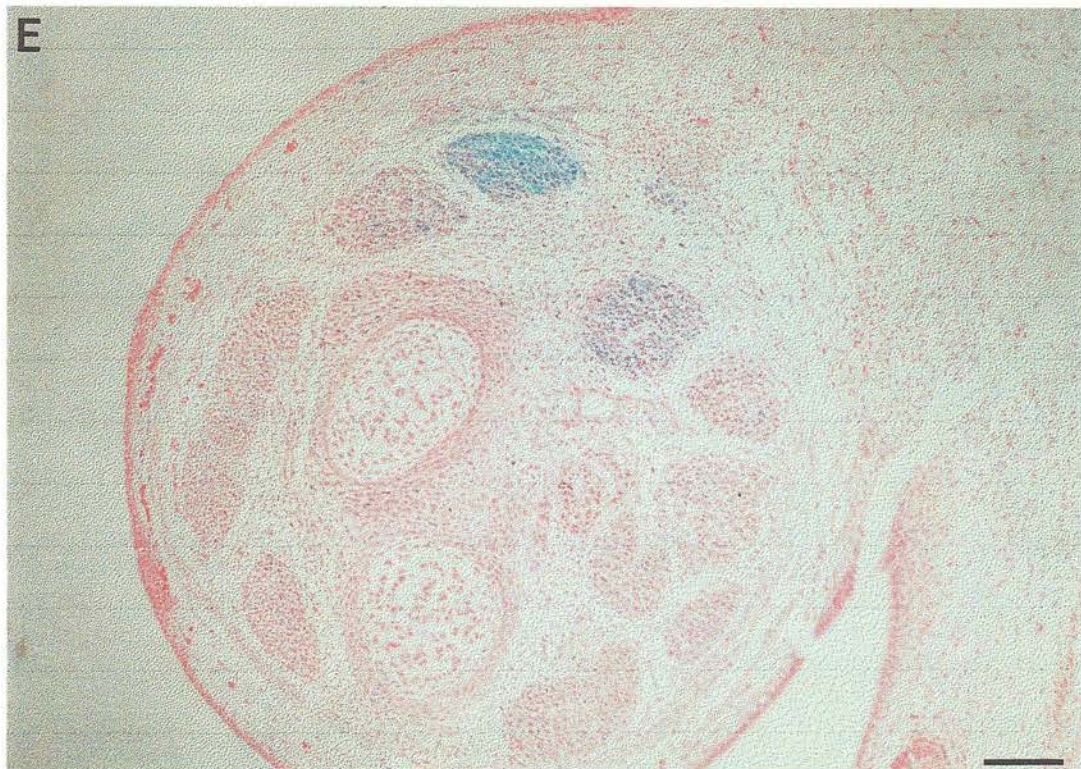
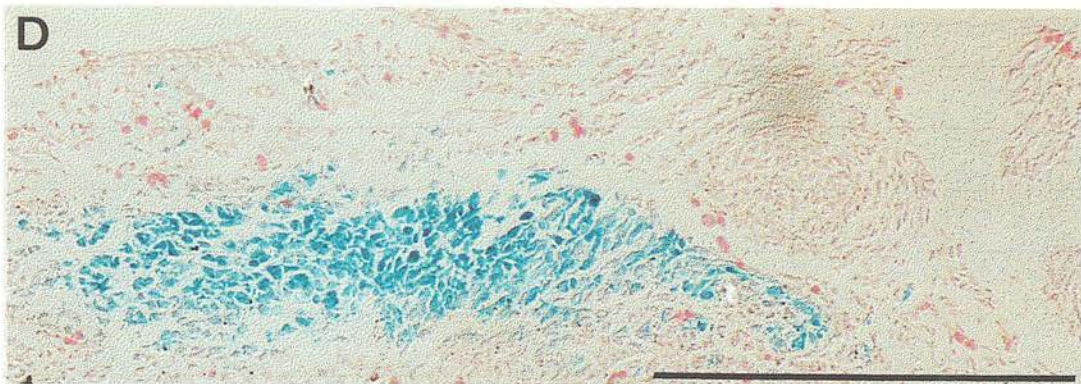
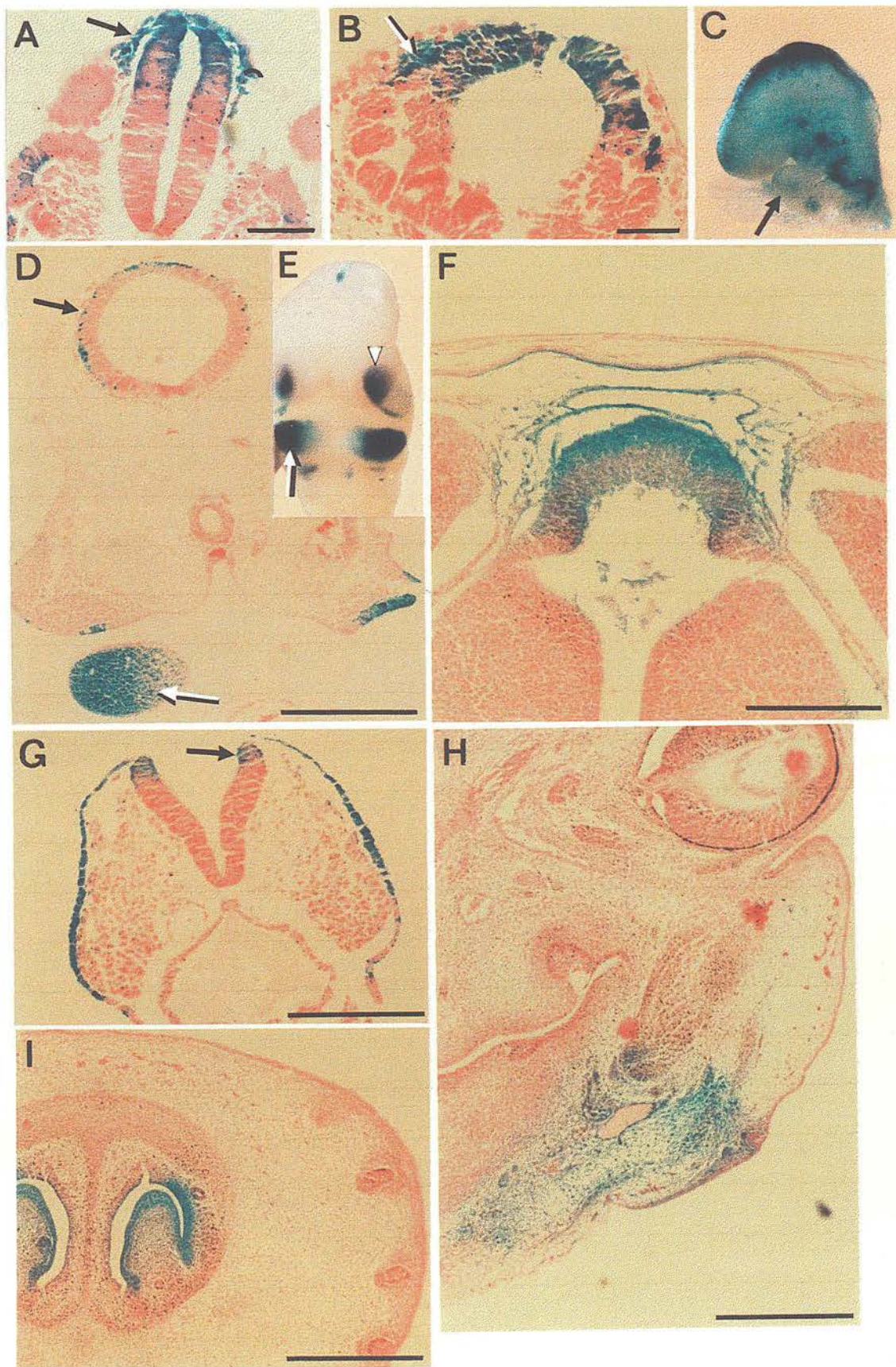


Figure 5.3

Aspects of $\Delta H5$ expression elsewhere in transgenic mice. See text for details.

- A. The arrow points to migrating neural crest in a transverse section of an E9.5 trunk.
 - B. The arrow shows migrating neural crest in a similar section at the level of the mesencephalon.
 - C. A whole E9.5 head. Most of the blue staining in the head is epithelial (see G). The arrow indicates the mandible, where blue staining is just appearing.
 - D,E. An E11.5 head. The black arrow in D shows expression in the neuroepithelium of the mesencephalon. The white arrow indicates the mandible. E. - Arrowhead shows blue staining in the lateral nasal epithelium (visible in D). The white arrow indicates the mandible.
 - F. E14.5 pineal gland.
 - G. The arrow indicates staining in the dorsal neural fold of the head prior to closure.
 - H. E14.5 frontal section of the mandible. Most staining is in the lateral connective tissue of the jaw, but part of the masseter muscle is stained.
 - I. Staining in the internal nasal epithelia at E14.5. Whisker follicles are negative.
- Scale bars = 10 μ m (A,B); 100 μ m (D-I).



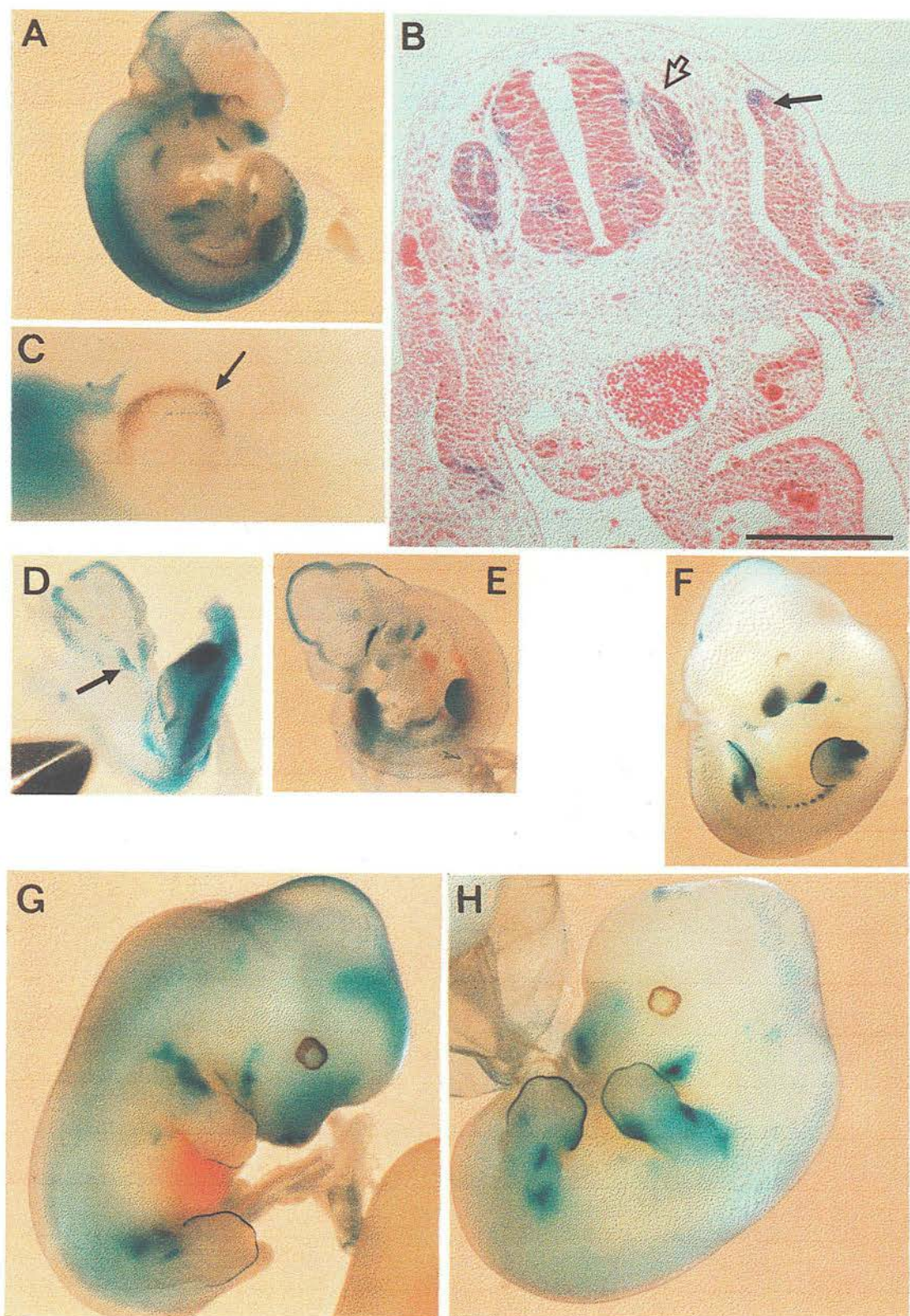


Figure 5.4

Expression of the transgene in other lines of mice.

A, B, C - ΔH6. A=E11.5, whole embryo. B is a transverse section at the level of the fore-limb. The open arrow indicates the dorsal root ganglion and the closed arrow the dorsal lip of the dermamyotome. Scale bar = 150μm. C is an enlargement of A, showing weak staining in the dorsal neural retinal cells.

D,E. - ΔH1. A=E8.5, the arrow shows staining in rhombomere 4. E=E10 whole embryo

F - E11.5 ΔH5, for comparison.

G - ΔH37, E13 whole embryo.

H = ΔH8, E13 whole embryo.

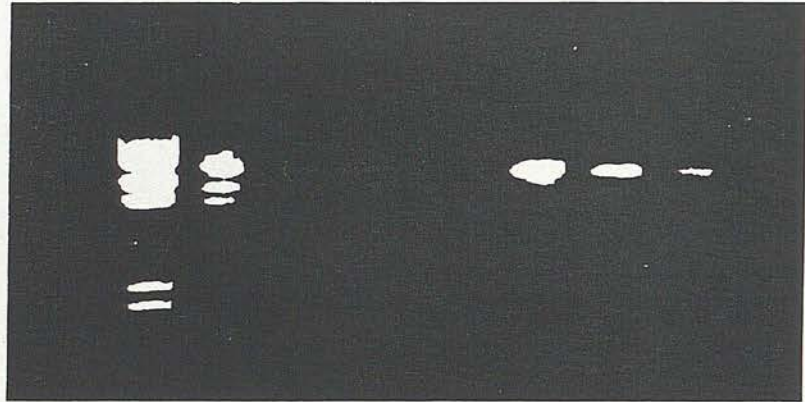


Figure 5.5

Isolation of the lacT insert.

Aliquots of serial dilutions of the lacT insert, run on a gel after isolation, show that the 15kb insert DNA was intact.

Size markers were the λ /HindIII fragments (left hand lanes)

lacT transgenic mice:

The creation of transgenic mice with the lacT insert provided an opportunity to confirm the observation (Chapter 4) that there were regulatory sequences in pH7lacT, absent from pH7lac Δ 3', which negatively regulated expression of *lacZ*.

Following the protocols described in Chapter 2, I digested 20 μ g of pH7lacT with *Not*I at 37°C for 2 hours, then with *Sfi*I at 50°C for 2 hours. Control digestions using either *Not*I or *Sfi*I individually showed that either enzyme would linearise 20 μ g of plasmid completely in 120 minutes.

After digestion, the 15kb insert band was separated from the 4kb pGEMEX band on a 0.8% LMP agarose gel. The gel was examined using low intensity U.V. light and the insert band cut out. The gel-slice was digested with agarase according to the manufacturers instructions and pure insert was isolated using Elutip-d columns.

The DNA was resuspended in filter-sterilised 'injection TE' (10mM Tris, 0.1mM EDTA, pH7.4) using Microcon tubes, then dialysed overnight.

DNA was stored in sterile, washed, dust-free Eppendorfs. 1 μ l was run on a 1% agarose gel, which showed that the insert had been successfully isolated. Serial dilutions of insert were run on a gel with known concentrations of λ /HindIII fragments and the concentration of insert estimated. It was 92ng/ μ l. This was diluted to 3ng/ μ l in injection TE (Figure 5.5).

This DNA was microinjected into the male pronucleus of fertilised mouse oocytes by Alasdair Mackenzie, as part of his ongoing analysis of *Msx1* promoter regions. This analysis is very preliminary; only two transgenic embryos have been produced to date. Both were fixed and stained at E11.5. The most obvious difference between these embryos and equivalent Δ H mice is the drastically lower expression level of the lacT transgene - blue staining is only detectable after several hours in X-gal. The expression pattern appears similar to Δ H mice - in particular, the expression in limbs is identical, with expression in the anterior myogenic domain, but none in distal mesenchyme - i.e. there is still no evidence of an inductive response to the AER signal. This is a preliminary indication that there are extra negative regulators in the lacT insert, not present in lac Δ 3', but that they are not tissue-specific.

CHAPTER SIX

Investigating the control of $\Delta H5$ transgene expression in experimental situations:

Introduction:

The pattern of expression of the transgene in several lines of mice shows strong similarities to elements of the expression pattern of *Msx1* (Chapter 5). It seemed likely that some of the differences were due to the transgene not containing the whole of the *Msx1* promoter (rather than being due solely to position effects). I decided to investigate the behaviour of the transgene under experimental conditions in order to investigate the interactions which could affect its expression, and to see how these differed from those which affected expression of endogenous *Msx1*. Whether the 4.7kb promoter contains elements necessary for reinitiation of expression in response to the AER signal remained an unanswered question.

$\Delta H5$ tissue was used throughout these experiments.

Experiment 6.01:

$\Delta H5$ tissue does not respond to the AER signal in a manner analogous to endogenous *Msx1*

Following the methods of Davidson *et al.* (1991), proximal mesenchyme was taken from E11.5 (W4/W5) transgenic hind-limbs and grafted beneath the AER of a stage 20 chicken fore-limb *in ovo*. After overnight culture (16-18 hours), the embryo was fixed and stained.

All grafts (7/7) were speckled with extremely variable numbers of blue cells. There was no widespread induction of the transgene in *Msx1*-expressing mesenchyme. Grafts which lay proximal to the Progress Zone also showed similarly variable numbers of blue cells (Figure 6.3 A,B,C).

This result was interpreted easily. Transgenic mesenchyme from the proximal/central region of the E11.5 hind-limb was not completely negative for β -Gal activity (in contrast to the endogenous gene). The expression pattern in the hind-limb at this stage (Figure 5.1 N), suggests that there was a high probability of including blue (myogenic) cells from the anterior and posterior regions. When grafted under the chicken AER, it appears that these blue cells maintain their expression, whereas the other clear mesenchymal cells do not start to express i.e. the transgenic tissue is unaffected by the graft.

This interpretation was confirmed as follows:

(i) By cutting proximo-central mesenchyme from the E11.5 hind-limb and staining immediately - 10/10 pieces of tissue had at least one blue cell, and as much as a quarter of the tissue could be stained. In the same experiment, 3 pieces of tissue were grafted into the flank mesenchyme of stage 20/21 chickens at a level just anterior to the fore-limb - a region which expresses neither the transgene nor *Msx1*. After overnight culture, the embryos were fixed and stained. All three grafts were speckled with small numbers of blue cells.

Mesenchyme from the distal handplate of W6 limbs is negative for transgene expression. The transgene cannot be reinitiated, even though the tissue is still open to signals which induce endogenous *Msx1*.

Experiment 6.01 suggests that expression of the transgene in the mouse tissue is unaffected by the AER, and is maintained in the absence of inductive signals. It is unlikely that the population of blue cells in tissue at time zero turns off *in ovo* and a separate small population of mesenchymal cells turns on. To clarify this result and to show there is genuinely no induction of transgene in any cells after grafting under the AER, I took tissue from the hand plate of the E11.5 fore-limb. This is completely negative for transgene expression at time zero (Figure 5.1).

Experiment 6.02

10 pieces of mesenchyme taken from the distal half of the E11.5 (W6) handplate - fixed and stained - 10/10 were completely negative for β -Gal

5 grafted underneath the AER of stage 20 chick limb, fixed and stained after overnight culture - 5/5 were completely negative (Figure 6.3 D)

3 grafted proximally in the stage 20 chicken as above - 3/3 completely negative (Figure 6.3 E).

It was obviously necessary to demonstrate that the W6 handplate mesenchyme was receptive to signals which could induce *Msx* gene expression. The experiments of Davidson *et al.* (1991) had been performed using W4/W5 proximal mesenchyme. Intuitively, it was likely that developmentally young, undifferentiated, W6 handplate mesenchyme would be responsive to extrinsic signals; Newman *et al.* (1981) had shown that distal handplate mesenchyme from the stage 25 (=W5/W6) chicken limb was still developmentally plastic.

As a direct test of receptiveness to *Msx1* inductive signals, I grafted mesenchyme from the middle of W6 transgenic handplates both under the AER of stage 20 chicken wings, and under the anterior epithelium of stage 23 wings, and performed radioactive *in situ* on this tissue after 18 hours *in ovo*. The mouse tissue expressed the endogenous *Msx1* only within the domain of chicken *Msx1* (3/3 distal grafts, 2/2 anterior grafts) (Figure 6.4 A,B,C and D,E,F).

Analysis of expression pattern of the transgenic mice would suggest that the transgene can not respond to the AER signal. The above grafting experiments support this. This may be either because the 4.7kb of promoter lacks the AER response element, or because it has it, but that some higher order repression prevents it from activating the gene.

If the transgene is not suffering from the effects of higher order repression ('silencing'), it may be possible to induce it in other areas of the body.

The most likely areas to get *de novo* induction of the transgene are those areas of the chick which correspond to transgene-expressing areas in the mouse. The limb provides two discrete areas where it may be possible to get induction of the transgene - the early mesenchyme and the later blue myogenic regions

To define where and when to graft into the chicken to expect to induce the transgene, I tried to define which chicken limb stages were equivalent to which mouse limb stages. Limb shape is the easiest way to do this, and in terms of developmental processes occurring, and genes being expressed, is reasonably accurate.

Hence:

Chick stage	Mouse limb stage	
=<19	=<W1	Chick AER appears
20	W2	
21/22	W3	Mouse AER appears
23	W4	
24/25	W5	
26	W6	
etc.		

Immigration of muscle cells, condensations, cell death etc. occur at similar equivalent developmental stages in mice and chicken. The major difference between the two at these stages is the early induction of the chicken AER compared to that of the mouse.

Distal grafts from experiment 6.02 had ended, at fixation, with transgenic tissue in a stage 24/25 Progress Zone which was expressing *Msx1*, but, were it a transgenic mouse limb of the same shape, would not show β -Gal activity. β -Gal is detected throughout the mesenchyme of the mouse limb until W4, which is the equivalent of a stage 23 chicken limb. I reasoned that grafting transgenic mouse tissue into very early chicken limbs so that at fixation after 18 hours the limbs were still of stage 23 or less may be a way to demonstrate induction of the transgene.

Experiment 6.03

Non-expressing Δ H5 handplate mesenchyme cannot reinitiate transgene expression when grafted into the very early chick wing bud:

Mesenchyme was obtained from the distal handplate of 11.5d Δ H5 mice (W5/W6). Representative pieces were fixed and stained immediately and were always negative. In 3 experiments, 18 pieces of tissue were grafted as distally as possible in to the limb buds of stage 17/18 chickens.

After 16 hours culture, the embryos were fixed and stained. All chickens were stage 23 or younger, the youngest was stage 21.

All grafts were completely negative (Figure 6.3 F,G).

All were in areas which would expect to express the transgene. 8 grafts were just under the AER in areas which definitely express *Msx1*. 7 were marginal to the distal domain of *Msx1* expression. 3 were proximal to the domain of *Msx1* (Fig. 6.2).

There was therefore no induction of the transgene when grafted into the very young chicken wing. I next attempted to induce gene expression by grafting into the anterior myogenic domain of older limbs. This domain is visible in mice from W5 to W14 at least - i.e. in chicken from stage 24 onwards. Grafts which, at fixation, were in the anterior zeugopod of stage 25-28 chickens might therefore have β -Gal activity.

Experiment 6.04

Non-expressing Δ H5 handplate mesenchyme cannot reinitiate transgene expression when grafted into the anterior marginal mesenchyme of the limb:

Handplate mesenchyme was taken from the E11.5 mouse fore-limb as above. Representative pieces were fixed and stained and were negative for the transgene.

11 grafts were performed into the anterior margin of stage 22 and 23 chick wings. After overnight culture these were near the anterior zeugopodal margin of the wings at stages 25/26/27, within the domain

which would be expected to express the transgene and at least partly within the anterior domain of *Msx1* expression (Fig. 6.2).

Upon fixing and staining, 11/11 were completely negative for β -Gal activity (Figure 6.3 H).

Hence not only was there no response of the transgene to an AER signal, there was no response to any signals which may cause expression of the transgene in the early limb bud or the differentiating myocytes.

I then showed that failure of reinitiation was not a consequence of any mouse/chicken incompatibility.

Experiment 6.05:

Non-expressing Δ H5 handplate mesenchyme does not reinitiate transgene expression when grafted into cultured mouse limbs or facial processes:

Distal handplate mesenchyme was taken from W6 fore-limbs and grafted into;

- (i) 10.5 day mouse limbs in culture (2);
- (ii) The anterior of 11.5d mouse limbs in culture (4);
- (iii) Mandibular mesenchyme of mouse heads in culture (16);
- (iv) Maxillary mesenchyme of mouse heads in culture (8).

For culture conditions, see below

Grafts were left 18 hours before fixing and staining. All 30 grafts were completely negative for transgene expression. Grafting into transgenic mandibles shows that the grafts were in transgene-expressing regions (Figures 6.2; 6.3 I,J,K).

Figure 6.1:

Experiment 6.05 - Experimental procedure

W6 handplate mesenchyme was taken from limb bud and grafted distally into the wing. 10.5 day mouse limbs in culture were fixed and stained for β -Gal activity. 11/11 were completely negative for β -Gal activity.

$\Delta H5$
W6 fore-limb

Graft site in chick

After overnight
culture

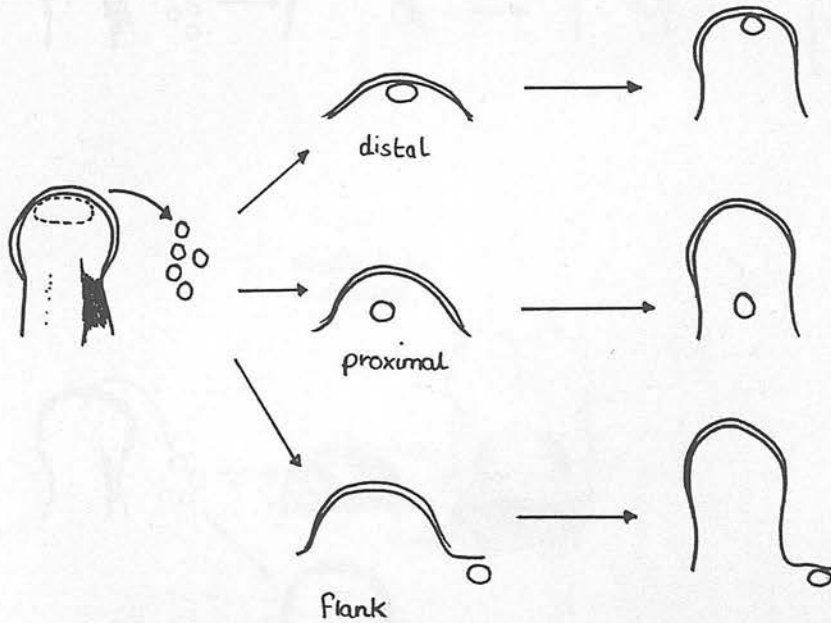


Figure 6.1:

Experiment 6.02 - Experimental procedure

W6 handplate mesenchyme does not induce *lacZ* expression when grafted distally into the stage 20/21 wing bud, proximally into the stage 22 wing bud, or into the flank mesoderm anterior to the wing.

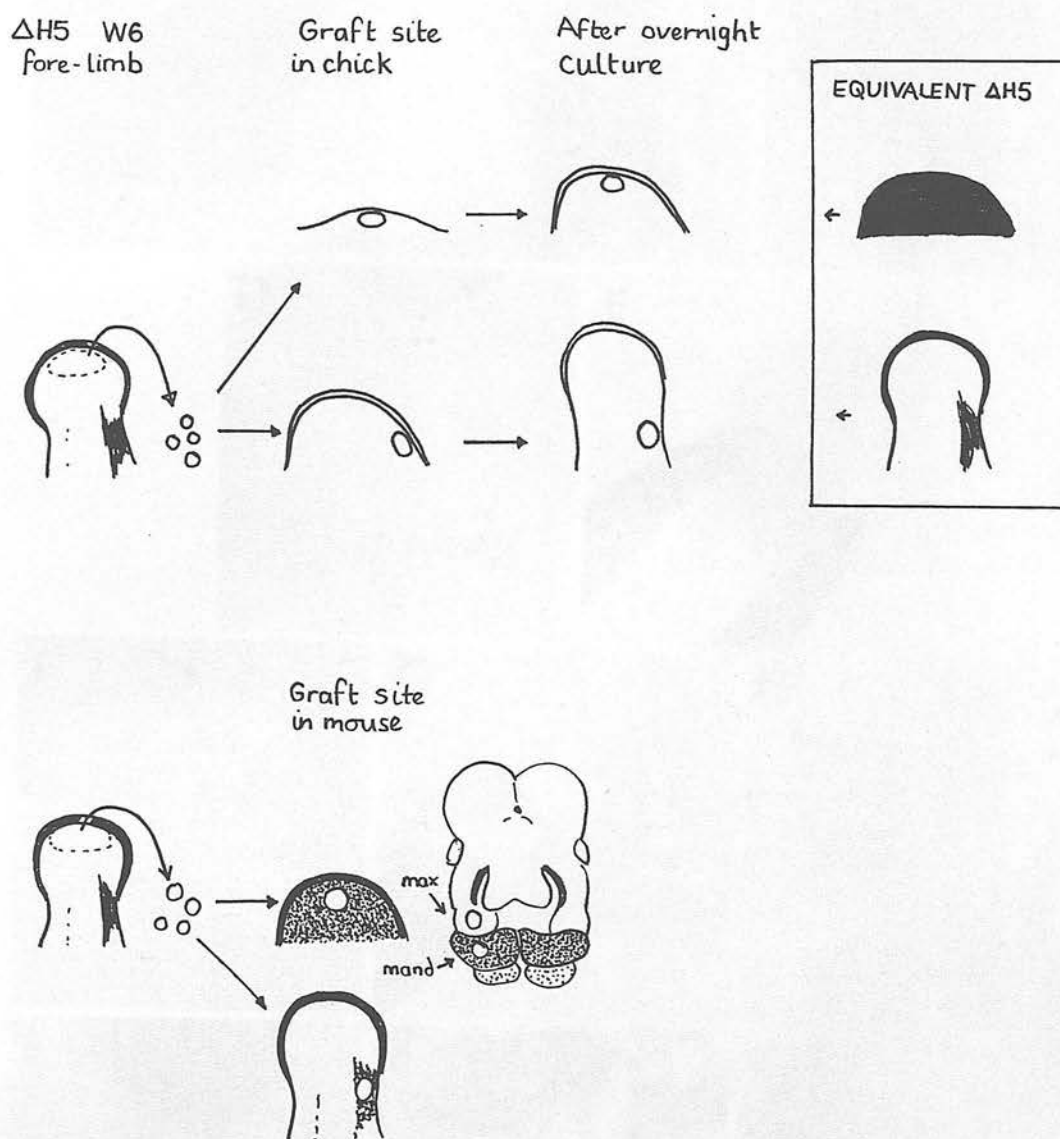


Figure 6.2:

Experiments 6.03, 6.04 and 6.05

W6 fore-limb handplate mesenchyme does not reinitiate transgene expression if grafted into the early chick or mouse limb bud, anteriorly into chick or mouse wings, or into the mouse maxilla or mandible.

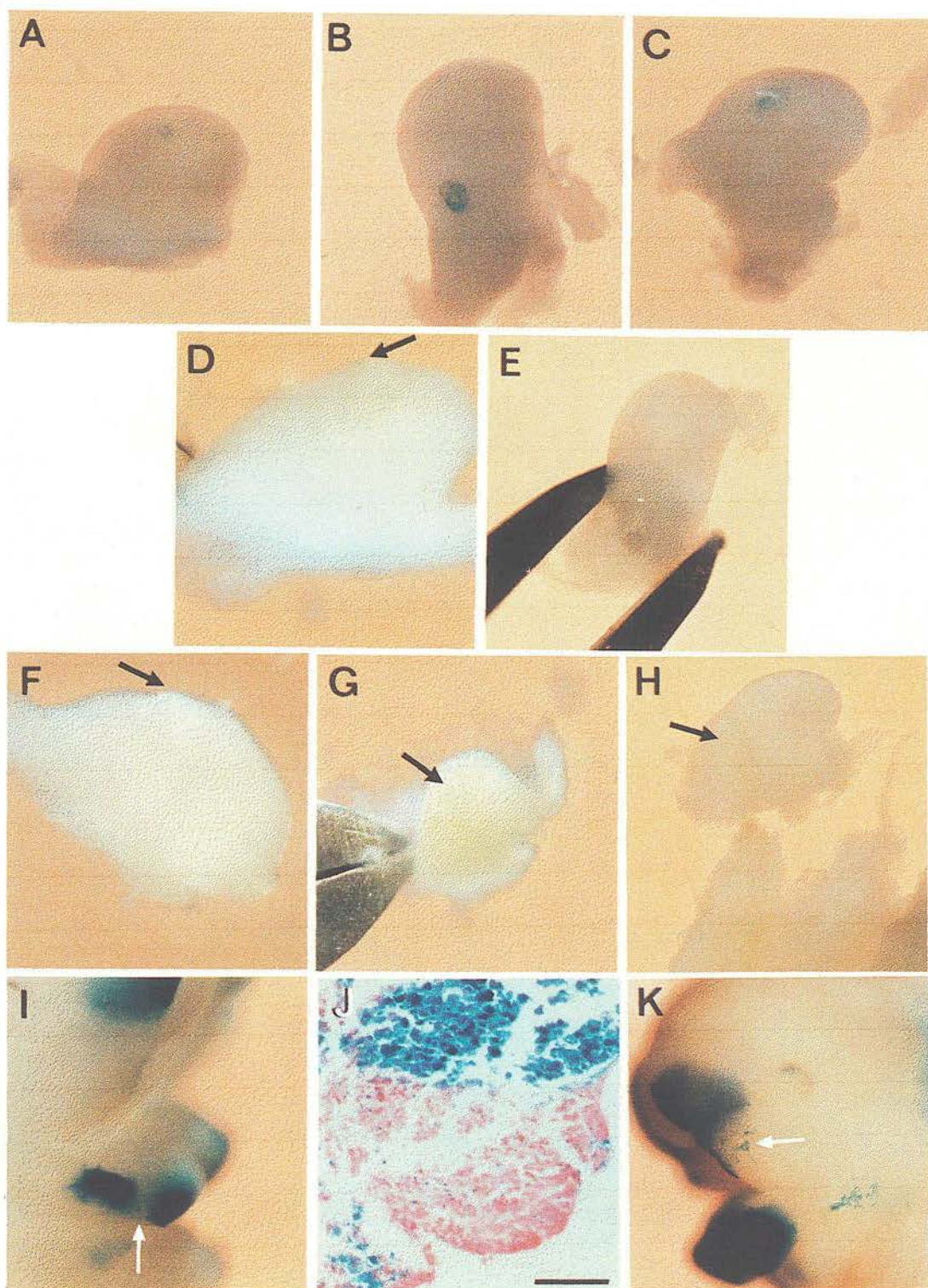


Figure 6.3

A, B, C. Experiment 6.01 - E11.5 Δ H5 hind-limb tissue grafted into chicken limbs for 18 hours. A and C are distal grafts, B is proximal.

D, E. Experiment 6.02 - Δ H5 handplate mesenchyme grafted distally, D, or proximally, E.

F, G. Experiment 6.03 - Δ H5 handplate mesenchyme grafted distally into stage 18 chicken wings for 18 hours.

H. Experiment 6.04 - Δ H5 handplate mesenchyme grafted anteriorly into the chicken wing, 18 hours.

I, J, K. Experiment 6.05 - Δ H5 handplate mesenchyme grafted into the mouse mandible (I, J) or the maxilla (K). The scale bar represents 50 μ m. These grafts were performed into transgenic heads.

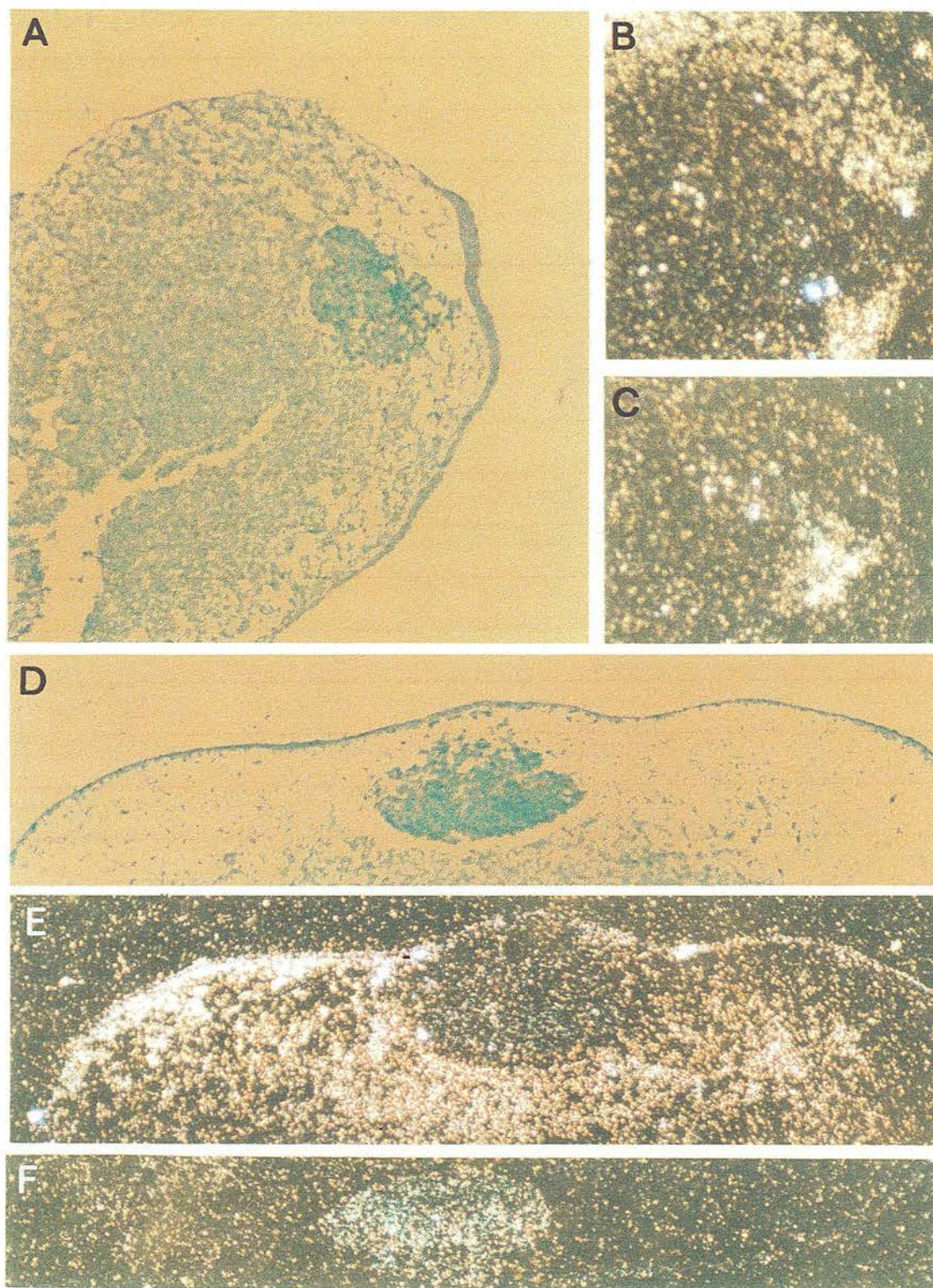


Figure 6.4

W6 handplate mesenchyme expresses *Msx1* within the chicken *Msx1*-expressing domain, whether grafted underneath the AER (A, B, C) or anteriorly (D, E, F).

In situ data on tissue sections. A and D are bright-field. B and E represent chicken *Msx1*, C and F are mouse *Msx1*.

Expression of pH7lac Δ 3' and pH7lacT in 10T $^{1/2}$ cells is not induced by placing them in these alternative locations:

The objections to Experiments 6.01 and 6.02 - that the grafted tissue was, at fixation, in a domain of the limb which would not be expected to express the transgene - could also possibly applied to the grafting experiments in Chapter 4. I had never observed convincing induction of pH7lacT or pH7lac Δ 3' in any grafts, even those which had, by accident, ended up in early limb buds or more proximal-anteriorly.

However, to deliberately check that that the problem with the experiments in Chapter 4 was not just that I had been attempting the wrong grafts, I performed some more grafts with the 10T $^{1/2}$ cell lines which had stably integrated pH7lacT or pH7lac Δ 3'.

Experiment 6.06

Balls of cells of the 10T Δ 3 line (mixed population of cells which have stably integrated pH7lac Δ 3') were grafted either into the anterior margin of stage 23 chick wings or distally into stage 17/18 wing buds.

They were fixed and stained after 16 hour culture. One plate of cells fixed and stained at without grafting showed low levels of expression at least in up to 1% of cells.

2/2 'early' grafts and 2/2 anterior grafts each showed up to 10 blue cells, at varying levels of expression. Blue cells were distributed apparently randomly throughout the graft. The number of blue cells is consistent with there being no difference between grafted and ungrafted cells (Fig. 6.5 A,B).

1/1 graft into the anterior margin of an 11.5d mouse limb in culture and 1/1 graft into the mandible of an 11.5d mouse head also showed the same random speckling of up to 10 blue cells (Fig. 6.5 C,D).

Experiment 6.07

As experiment 6.06, but using the 10T14 cell line (clonal cell line containing pH7lacT)

All the grafts (4/4 anterior, 3/3 'early') were either negative or showed one or two blue specks on staining. A 60mm plate of confluent cells showed less than twenty blue cells, even after overnight staining (Fig. 6.5 E,F).

I also grafted these cells into the anterior margin of E11.5 Δ H5 mouse fore-limbs in culture. After 16 hour culture, 2/2 grafts were negative (Fig. 6.5 G).

One graft into the lateral distal mandibular mesenchyme of an 11.5d Δ H5 mouse head in culture was also negative after 18 hour culture.

Conclusions:

There was therefore no widespread induction of *lacZ* in the lines of 10T¹/₂ cells even when grafted into areas of the chicken which might be expected to induce the transgene. Grafts into cultured mouse tissue showed this was not a problem of mouse/chicken tissue incompatibility.

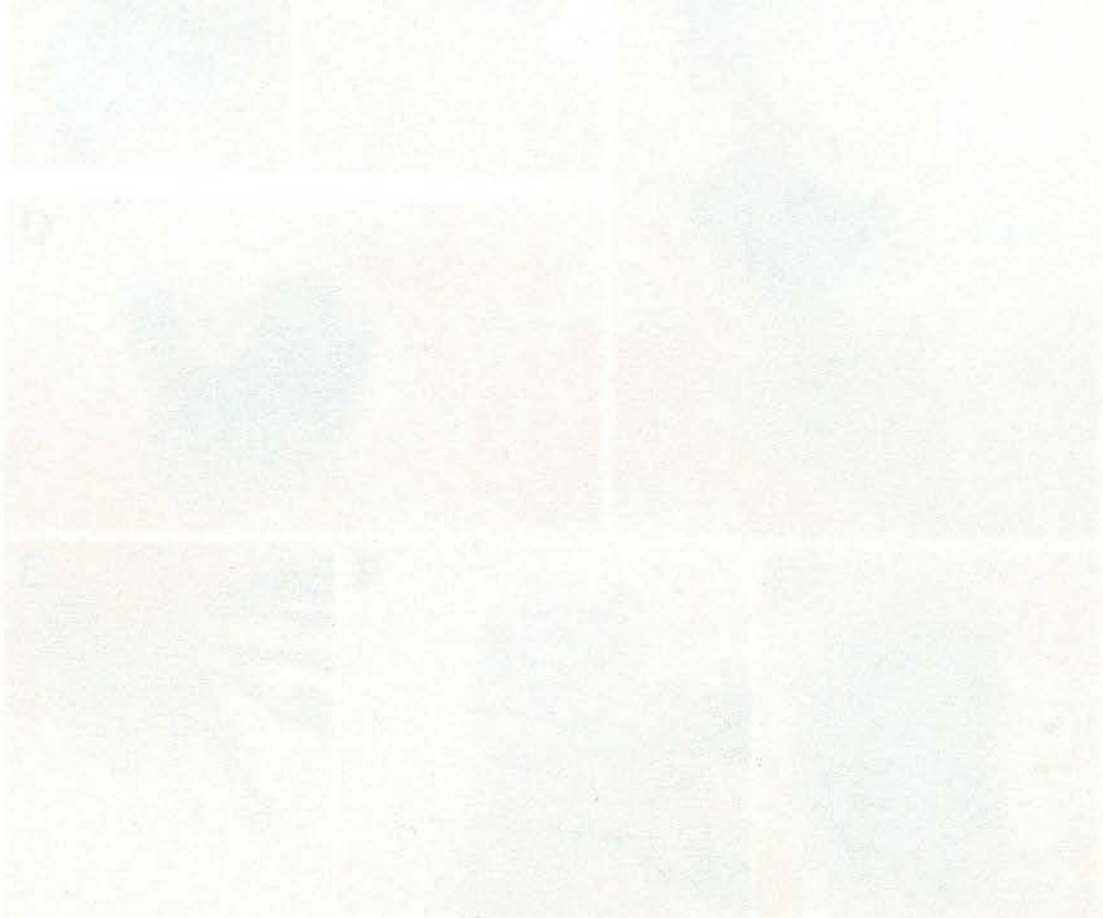


Figure 4.5
10T¹/₂ cells grafted into chicken tissue. The sections show lacZ induction in the grafted cells. The sections are labeled A through L in the top left corner of each panel. The panels show varying degrees of blue staining, indicating lacZ induction, against a pink background of tissue. Panels A, C, E, G, I, and K show significant blue staining, while panels B, D, F, H, J, and L show less staining or none at all.

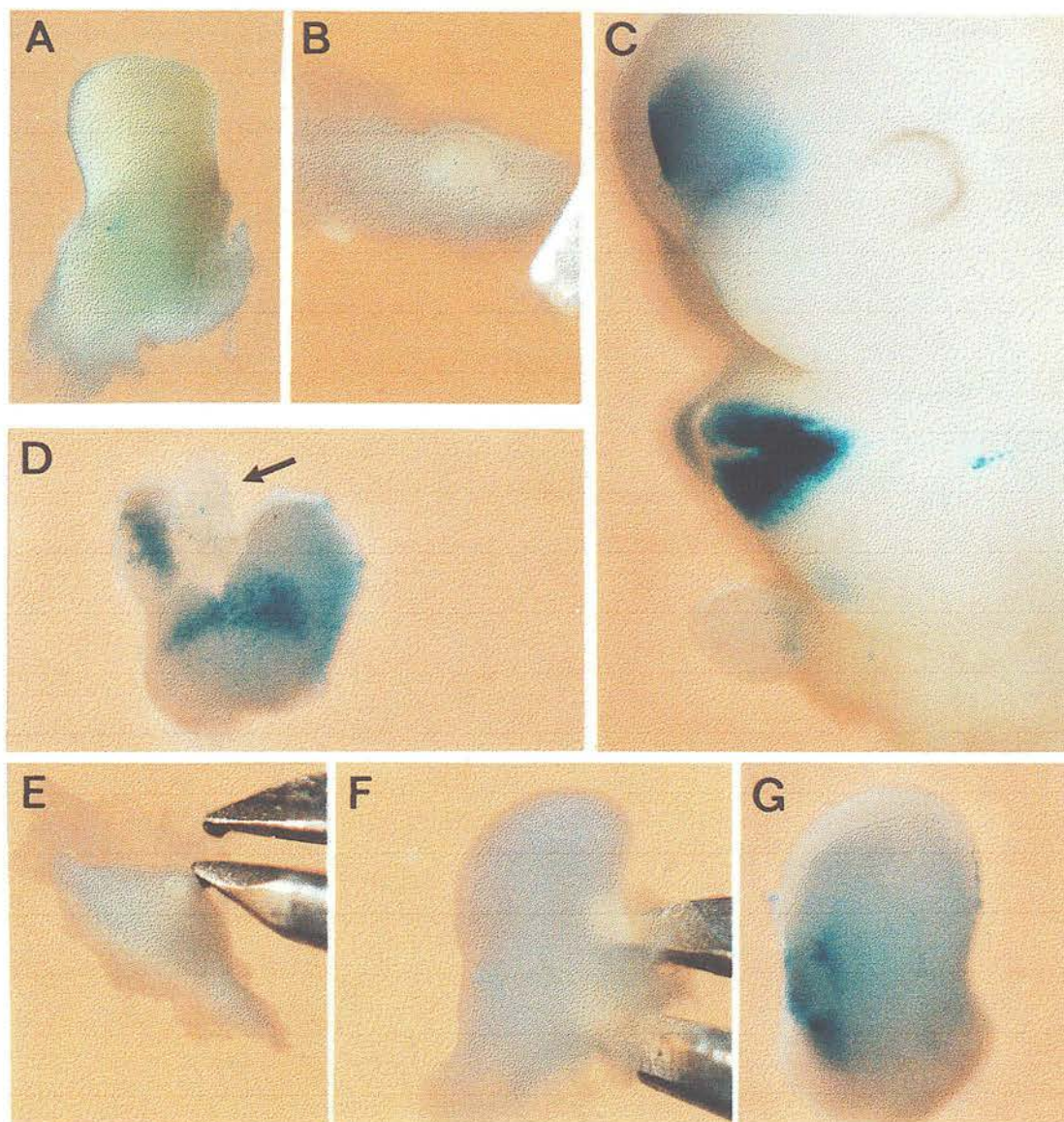


Figure 6.5

A-D. Experiment 6.06. Expression of *lacZ* in 10TΔ3 cells is not induced within 18 hours when grafted anteriorly into the chick wing (A) or into the very young chick wing (B), into the mouse mandible (C) or limb(D). The limb in C has been broken to expose the graft (arrow). C and D were grafts into transgenic tissue, to show that the grafts were in areas which express the transgene.

E-F. Experiment 6.07. 10T14 cells do not induce *lacZ* expression when grafted into the very young chick wing (E), anterior chick wing (F) or anterior transgenic mouse limb (G).

Behaviour of transgenic tissue in culture:

As part of the investigation into the extrinsic factors which modulated expression of the transgene, I needed some indication of the 'ground state' of the tissue in the absence of inductive or repressive influences. $\Delta H5$ tissue was cultured to investigate the degree of cell autonomy shown by the transgene.

Gibco CMRL1066 medium was used, at 37°C and 5% CO₂ in a humidified environment on Falcon 35mm tissue culture dishes. To be certain that there were no inductive growth factors etc. present, one would use a defined complete medium which contained no serum. Prior to looking for a suitable defined medium, I performed some preliminary experiments using CMRL1066. All experiments were performed both with and without 10% FCS. Results were always identical, but cells grew and adhered better in FCS. Because the presence of FCS did not visibly modulate expression of the transgene, the recipe of '1066+10%' was followed subsequently.

Experiment 6.08

Limbs survive and grow in culture:

(i) *11.5 day fore- and hind-limbs - whole limbs cultured with ectoderm, 24hours.*

The limbs grew slightly. Expression pattern was unchanged (Figure 6.6 B), with strong expression detectable in the AER and anterior mesenchyme.

(ii) *11.5d fore- and hind-limbs - whole limb mesenchyme, 24hours.*

Whole limbs were placed in 0.2% trypsin at 20°C for 20 minutes until the ectodermal jacket could be removed, and the mesenchyme cultured. Mesenchyme adhered to the dish and cells started to grow out. The limbs lost much of their shape but the anterior patch of mesenchymal expression was still recognisable (Figure 6.6 A).

(iii) *10.5d fore-limb (stage W4) - whole limb in culture, 24hours.*

The mesenchyme of E10.5, W4, limbs was strongly positive for *lacZ* activity.

Whole limbs, with ectoderm, were isolated and cultured. After 24 hours, limbs stayed completely blue - their shape did not noticeably change and they did not become '11.5d' limbs.

(iv) *10.5d fore-limbs - trypsinised and de-epithelialised, 24hr.*

W3/4 fore-limbs were trypsinised and de-epithelialised as before.

Cultured mesenchyme adhered and grew. All limbs (>10) were largely negative for β -Gal activity after 24 hours, but had a discrete speckled patch of blue cells, in random positions either in the middle or on the edge, depending on the orientation in which the limb had sat down (See Figure 7.3).

Experiment 6.09

Expression in mesenchyme derived from the lateral plate is lost in the absence of an overlying epithelium, but expression in blue myogenic cells remains for at least 24 hours and may be cell autonomous:

(i) *11.5d (W6) fore-limb distal handplate mesenchyme, trypsinised and de-epithelialised, 24hr.*

200 μ m pieces of mesenchyme from the distal handplate of W6 fore-limbs were cultured. Cells grew out from the lump of tissue as a monolayer and remained completely negative for β -Gal activity (8/8) (Figure 6.6 C).

(ii) *11.5d fore-limb anterior mesenchyme - trypsinised and de-epithelialised.*

Mesenchyme was isolated from the anterior zeugopod of W6 fore-limbs. Representative pieces were fixed and stained immediately and all showed large patches of blue cells.

Other pieces were cultured, 24 hours, before staining. All (9/9) retained large patches of blue cells which grew out as a monolayer. (Figure 6.6 F).

Other cultures of these cells were maintained for up to a week. Greatly reduced numbers of cells showed β -Gal activity after 2-3 days in culture (Figure 6.6 G). After 5-7 days, when differentiating myotubes, adipocytes and chondrocytes are seen in the culture, none of the cells had detectable β -Gal activity (Fig. 6.6 H)

(iii) *10.5d (W3/W4) fore-limb distal Progress Zone mesenchyme, 24hours.*

Mesenchyme from the Progress Zone of W3/W4 fore-limbs was isolated. Representative pieces were fixed and stained immediately, and all showed strong β -Gal activity; other pieces were cultured for 24 hours.

On staining after 24 hours, they were either completely clear of β -Gal activity or showed some very faint uniform residual blue (Figure 6.6 D).

Experiment (iii), above, when compared with Experiment 6.08 (iii), suggested that expression of the transgene in W3/W4 Progress zone tissue may be dependent on a signal from the limb epithelium. It was possible however that there was a factor within the mesenchyme, not epithelially derived, which maintained the transgene in whole-limb cultures but was leached away in the absence of epithelium.

To test this possibility, the distal cap of ectoderm and mesenchyme was dissected from W3/W4 forelimbs, and cut in half so that a large surface area of mesenchyme would be exposed to the culture medium to allow leaching of mesenchymal factors. The fragments were cultured as above for 24 hours before fixing and staining.

(Figure 6.6 E) Mesenchyme which was in close proximity to the epithelium was blue (even cells which were in the monolayer exposed to the medium). Those cells which were not adjacent to the epithelium were clear of β -Gal activity.

The active epithelium need not include a morphologically distinguishable AER.

Conclusions:

11.5d tissue:

In culture, in the presumed absence of epithelial signals and signals from the rest of the body, non-expressing mesenchyme remains negative for β -Gal and expressing anterior (myogenic) cells remain positive for at least 24 hours.

The presence of outgrowing positive cells in culture suggests that the maintenance of expression in myogenic cells during this 24 hours requires little or no contact with other cells.

10.5d tissue

10.5d , W3/4, Progress Zone mesenchyme, which expresses the transgene but contains no myogenic cells, loses β -Gal activity within 24 hours if removed from its overlying ectoderm.

The patch of blue cells which remains in the de-epithelialised whole limb after 24 hours in culture probably represents a population of myogenic cells.

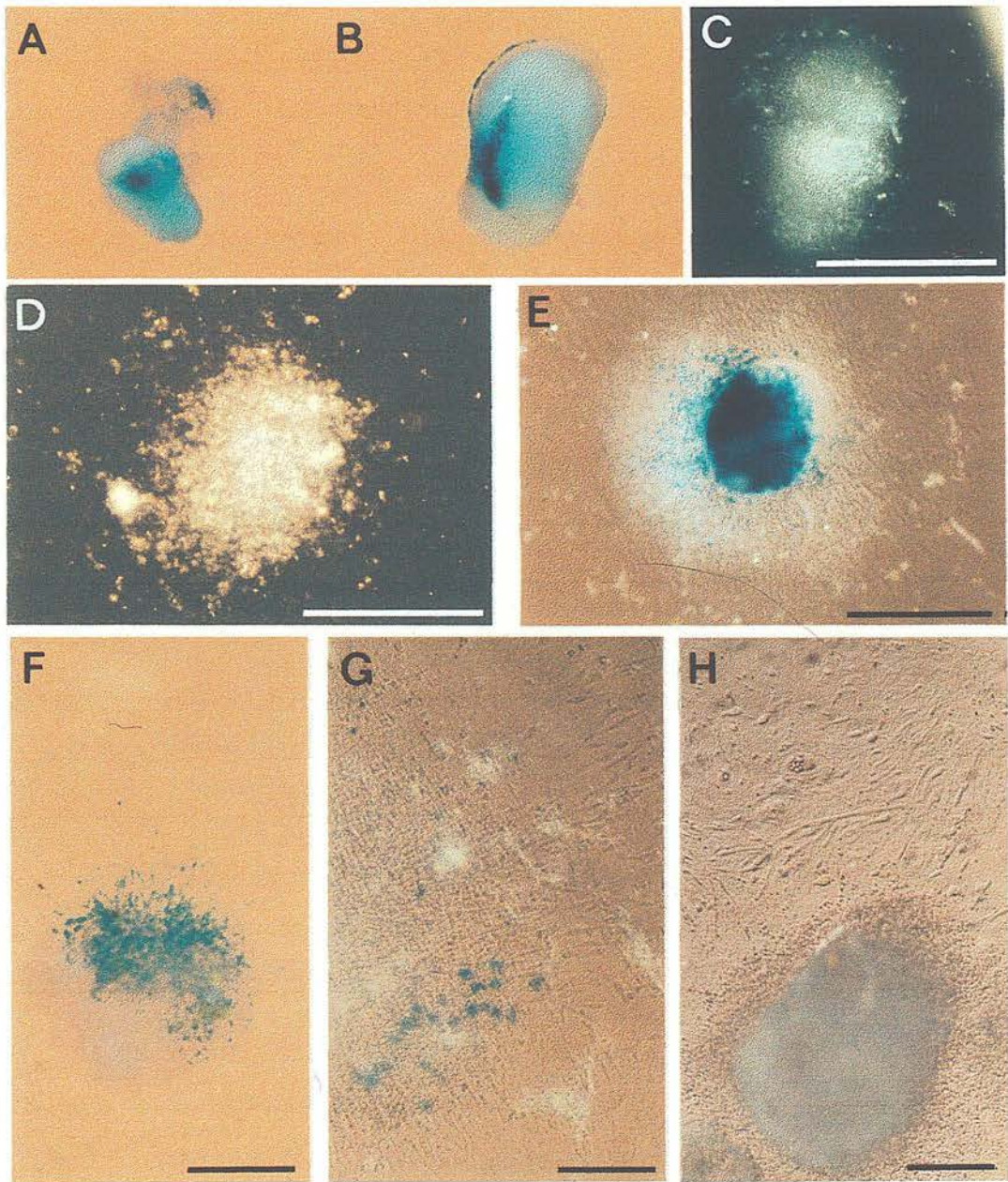


Figure 6.6

Experiments 6.08 and 6.09

A. E11.5, W5, de-epithelialised whole limb mesenchyme, cultured 24 hours.

B. Whole W5 limb, cultured 24 hours.

C. W5 Progress Zone mesenchyme, cultured 24 hours.

D, E. E10.5 (W4) Progress Zone mesenchyme, cultured 24 hours without (D) or with (E) overlying epithelium.

F, G, H. Anterior W6 (E11.5) mesenchyme (myogenic) cultured 24 hours (F), 2.5 days (G) or 7 days (H)

Scale bars = 500 μ m

The behaviour of W6 anterior blue cells in grafts:

Experiment 6.09 (ii) and Experiment 6.08 (ii) suggested that expression of the transgene in the anterior myogenic cells of the W5+ fore-limb could be maintained for 24 hours in the absence of any specific signal from the anterior limb epithelium. To confirm that this was so, and to see if there were any negative regulators which could downregulate the transgene, I grafted these cells into various sites in the chicken and mouse (Fig. 6.7).

Experiment 6.10

Cells from the anterior of the W6 limb retain expression of the transgene for at least 24 hours, independent of their position in the embryo:

Mesenchyme was taken from the de-epithelialised anterior zeugopod of 11.5d, W5/6, ΔH5 transgenic mice.

3 grafts were placed under the AER of stage 20 chickens. After further 18 hours culture, all grafts were distal in stage 23/24 wings. All 3 showed large patches of blue cells (Figure 6.8 B).

3 grafts were placed proximally into stage 22/23 wings. After 18 hours these were in areas which would be expected to express neither *Msx1*, nor the transgene. All showed large numbers of blue cells upon staining (Figure 6.8 C,D).

3 grafts were placed into the flank mesoderm of a stage 20 chicken, anterior to the fore-limb bud. After 18 hours, 3/3 retained intense expression of the transgene (Figure 6.8 E).

As a positive control, 5 grafts were placed anteriorly into stage 23 wing buds, and stained after 18 hours.

Limbs were stages 26/27; 5/5 were strongly positive for β-Gal (Figure 6.8 A).

1/1 graft into the mandibular mesenchyme of a 11.5d mouse in culture showed strong β-gal activity after 24 hours (Figure 6.8 F).

Blue anterior cells can maintain expression in the chicken limb for at least $3\frac{1}{2}$ days, and can contribute to muscles which are different from those to which they would contribute in the mouse.

β -Gal activity in de-epithelialised W6 anterior cells was greatly downregulated after 3 days in culture, suggesting that *lacZ* expression was not as autonomous as the above experiment implied. I performed experiments to investigate whether these blue cells would maintain expression in the chicken for more than 48 hours and to see if they would differentiate into muscle.

Experiment 6.11

Anterior mesenchyme was isolated from W6 fore-limbs as before, and grafted proximally into stage 22 or 23 chicken wings.

One graft was fixed and stained after 18 hours and showed large numbers of blue cells as before. 5 grafts were fixed and stained after 48 hours. All grafts showed large clumps of blue cells. In two cases, there had been significant migration of cells distally from the graft (Figure 6.8 G). One of these grafts was wax embedded and sectioned (Figure 6.8 H). It showed that the cells were still morphologically undifferentiated, but blue cells had aggregated as a clump, away from the non-blue mesenchymal body of the graft, within the chicken's myogenic mass.

Six grafts were fixed and stained after $3\frac{1}{2}$ days. All showed clumps of blue cells. 3 showed a longitudinal alignment which suggested that they were contributing to muscle (Figure 6.8 I). These grafts were wax-embedded and sectioned. This confirmed that they had differentiated into myotubes within the chicken's differentiating muscle blocks (Fig. 6.8 J). The muscles which they contributed to were not necessarily those anterior muscles which they were fated to form in the mouse. There were very few blue cells away from the differentiating muscle blocks.

This result strongly suggests that expression in the blue cells is maintained for longer in the chicken limb than it is in culture, and so is not completely cell-autonomous. The maintenance factor does not however have to be a specific anterior signal.

It also confirms that most, maybe all, of the blue cells in the anterior of the W5/W6 mouse limb are myogenic. Lateral plate mesenchymal cells cannot differentiate into limb muscle.

Contribution of mouse myocytes to chicken muscle is an important result. Whilst not unexpected, it was previously unreported, and was *contra* Kieny (1987), who was unable to show that it could occur. It does agree with the recent data of Fontaine-Perus *et al.* (1995), who showed that mouse somites can, in grafting experiments, contribute to chicken limb muscle.

ΔH5 W6
Fore-limb

Graft site

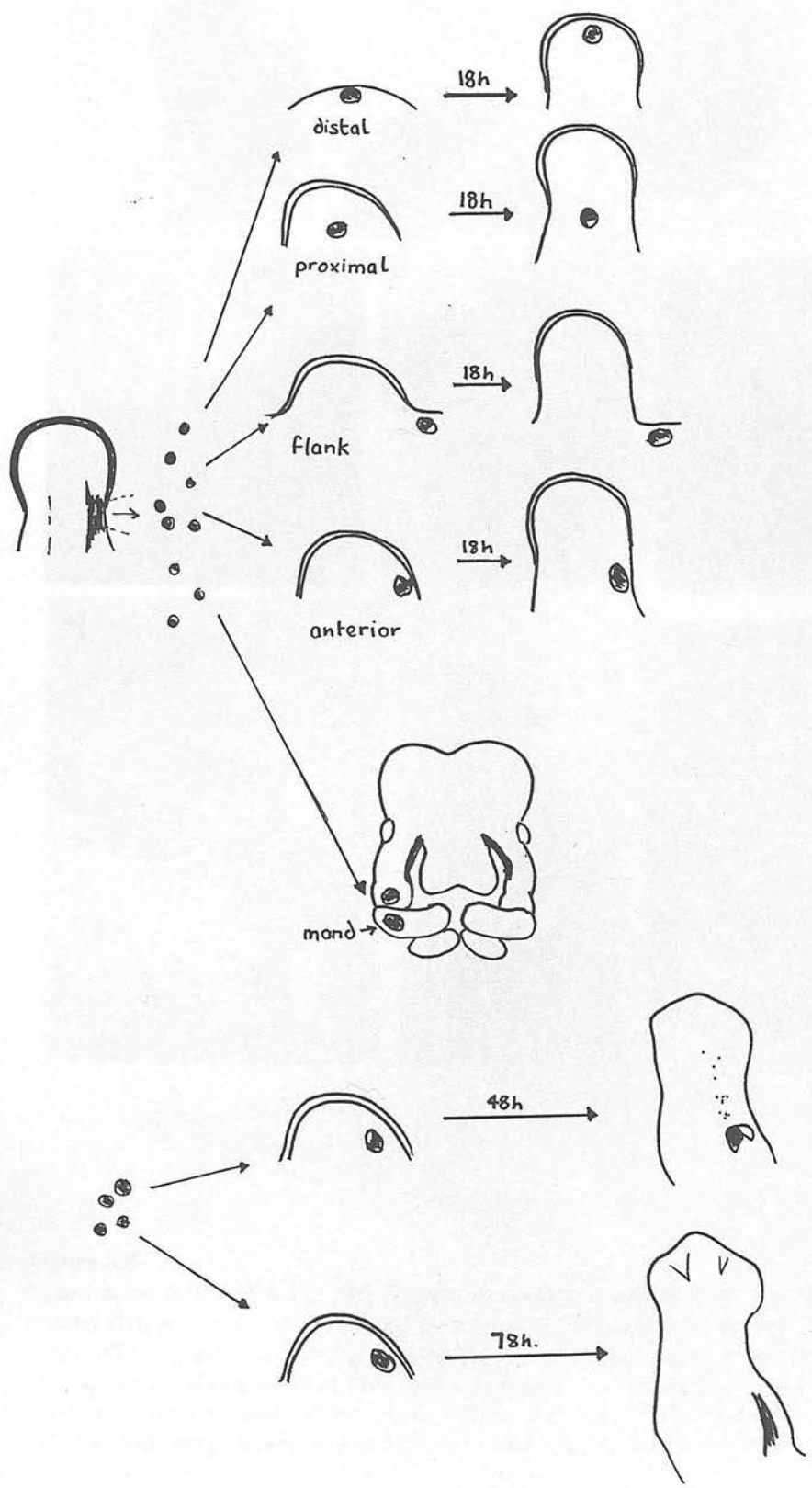


Figure 6.7:

Experiments 6.10 and 6.11

Transgene-expressing W6 anterior cells retain expression for 18 hours, independent of graft site, in mouse or chick, and can contribute to the chick musculature in 2-3 days. All grafts were to chick, except for mandibular/maxillary mouse grafts. Anterior is to the right.

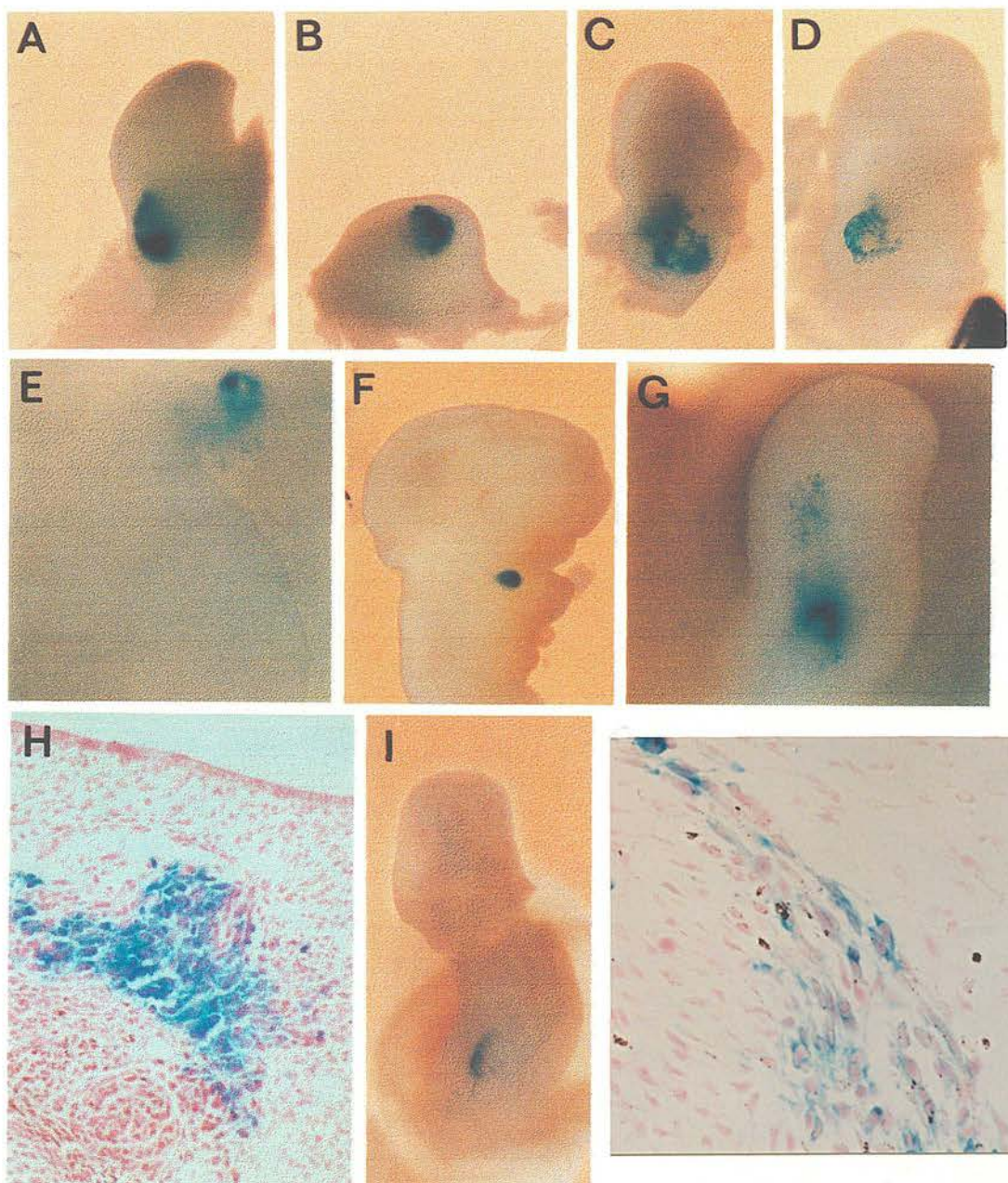


Figure 6.8

Experiments 6.10 and 6.11. W6 anterior mesenchyme grafted into chicken wings, anteriorly (A), distally (B), proximally (C, D), flank (E) or in to the mouse mandible (F). All remain blue after 24 hours. G was grafted proximally into the chicken wing and remained positive upon staining after 48 hours, with distal migration of blue cells. A section of a limb like G (H) shows blue cells clumped within the myogenic mass of the chick. I shows a graft stained 3.5 days after implantation anteriorly into the chick wing. J (a section of I) shows differentiating blue myocytes within the chicken muscle.

Modulation of transgene activity by grafting:

In vivo, Progress Zone tissue from the 10.5d fore-limb (W4), displays intense β -Gal activity, but will have lost it completely within 24 hours (W6). I decided to see whether expression of the transgene could be rescued by grafting 10.5d Progress Zone tissue either:

- (i) into the early chick wing bud;
- (ii) into the anterior mesenchyme of the stage 23 chick wing bud.

Experiment 6.12:

Expression of the transgene in E10.5 Progress Zone mesenchyme can be maintained experimentally for 24 hours:

Distal mesenchyme was taken from 10.5d, W4, Δ H5 fore-limbs.

Random pieces were selected, fixed and stained immediately - all were strong uniform blue. Random pieces were cultured for 24 hours as before.

Grafts were performed:

- (i) either distally or proximally into chicken wings stage 17-21;
- (ii) into the mesoderm of the flank at a level just anterior to the limb (as before) in chickens stage 20-24;
- (iii) into the anterior mesenchyme of limbs stage 22-24;
- (iv) into the proximal central mesenchyme of stage 23-24 wings.

(Figure 6.9)

All grafts were fixed and stained after 24 hours *in ovo*.

Results:

All cultured tissue was either *clear of β -Gal activity* or showed very faint residual blue

9/9 grafts were inserted into limbs of stages 17/18/19 and which therefore were in chicken limbs of ≤ 23 at fixation. All were *positive for β -Gal activity*, 6 very strongly so (Figure 6.11 A,B).

5 distal and 2 proximal grafts into limbs stage 20-21 which finished distally or proximally in limbs of stage >23 - <25 . 5/7 were *weakly positive* for β -Gal, 2/7 were completely *negative* (Figure 6.11 F,G).

6/7 grafts to the flank mesoderm were *completely negative*. 1/7 showed some faint blue.

6/8 grafts to the anterior limb mesenchyme, which finished the experiment in the anterior mesenchyme of limbs aged 25-27, were *positive* for transgene activity, 4 very strongly so. 2/8 were negative (Figure 6.11 C,D).

4/5 grafts into the proximal central mesenchyme which finished proximally in wings \geq stage 25 were *negative*. 1/5 showed faint blue (Figure 6.11 E).

In addition 4/4 grafts which rested on the surface of the limb were *negative or faintly blue*. For any graft which stuck out of the limb, the exposed section was never strongly blue.

Cells which were negative for β -Gal activity and sticking out of the limb were probably still alive until fixation (and therefore a genuine result). Milaire, 1965 stated that mesenchymal cells could survive when exposed directly to amniotic fluid.

Conclusions:

The primary result from this set of experiments was that *expression of the transgene can be rescued over the 24 hour period that it would normally turn off*, e.g. by grafting into a very young chick wing. The factors that are maintaining expression in this tissue cannot reinitiate expression in 11.5d non-expressing tissue.

Converting mouse limb stages to chick stages as above, W4 = stage 23, therefore the mesenchyme of the wing of any chick \leq stage 23 would be expected to express the transgene, but would fade out between stages 24-25 such that after stage 25 (W6) only the anterior myogenic cells would be expected to show β -Gal activity. Hence in the above experiments, it is seen that *the state of β -Gal activity in the grafts at the end of 24 hours reflects the state of transgene expression in the equivalent areas of the mouse at the same stage*. This result appears almost independent of the expression domains of the endogenous gene.

Maintenance of expression anteriorly in the older limbs is less reliable (2/8 grafts downregulated expression) so the above paragraph may not fully explain the results.

It would be predicted from the above that expression in 10.5d Progress Zone tissue would be maintained if grafted into the mandible, but not into the maxilla. I tested this hypothesis.

Experiment 6.13:

Grafting into the mandible only weakly rescues 10.5d Progress Zone expression:

Mesenchyme from the Progress Zone of W3/4 Δ H5 fore-limbs was either:

- (i) cultured for 24 hours;
- (ii) grafted distally into the mandibles of E10.5d mice;
- (iii) grafted distally into the maxillae of E10.5 mice.

Fixing and staining was carried out after 24 hours.

Results:

As before, cultured tissue showed almost no blue after 24 hours.

3/5 grafts into the maxilla were completely negative for β -Gal, 2/5 showed very faint blue (Figure 6.11 I)
4/4 grafts into the mandible were weakly positive. All were stronger than grafts to the maxilla, but none were as strong as those which had been grafted into very young chick wings (Figure 6.11 H).

2/2 grafts to the early mouse limb in culture also showed *lacZ* expression after 24 hours (Figure 6.11 J)

I will return to these results in the Discussion.

Inability to reinitiate transgene expression is a function of its downregulation:

Experiment 6.12 and 6.13 had suggested that expression of the transgene could be maintained by factors present in the early limb bud, the anterior myogenic regions of the bud or in the mandible, but Experiment 6.05 showed that these factors could not reinitiate expression of the transgene in negative W6 Progress Zone mesenchyme.

This raised the possibility that there were negative regulators specific to the W6 tissue which were not present at W4. Alternatively, the act of downregulation of expression may cause a change in the promoter which prevents reinitiation, or reinitiation may require factors which are not necessary for maintenance.

In these latter cases, it would be predicted that W3/4 Progress Zone mesenchyme which was allowed to downregulate expression for 24 hours in culture would not subsequently be able to reinitiate it when grafted into the early chicken wing, or anteriorly into the stage 23 wing.

This prediction was tested in some preliminary experiments.

Experiment 6.14

W4 Progress Zone tissue which is allowed to downregulate transgene expression in culture cannot subsequently reinitiate it when grafted into the chick wing

200µm pieces of 10.5d, W4 Progress Zone tissue was isolated and cultured overnight as before. After 24 hours, representative pieces were fixed and stained - all were either completely negative or very faintly blue. Other pieces were removed from the culture dish and grafted either into stage 17/18 chicken wings or anteriorly in stage 22 wings.

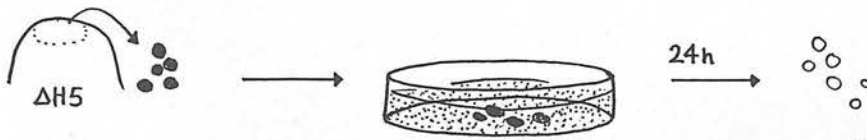
After further overnight culture, embryos were fixed and stained.

3/3 grafts underneath the AER of wing of stages 22/23 were completely negative for β-Gal activity (Figure 6.11 J).

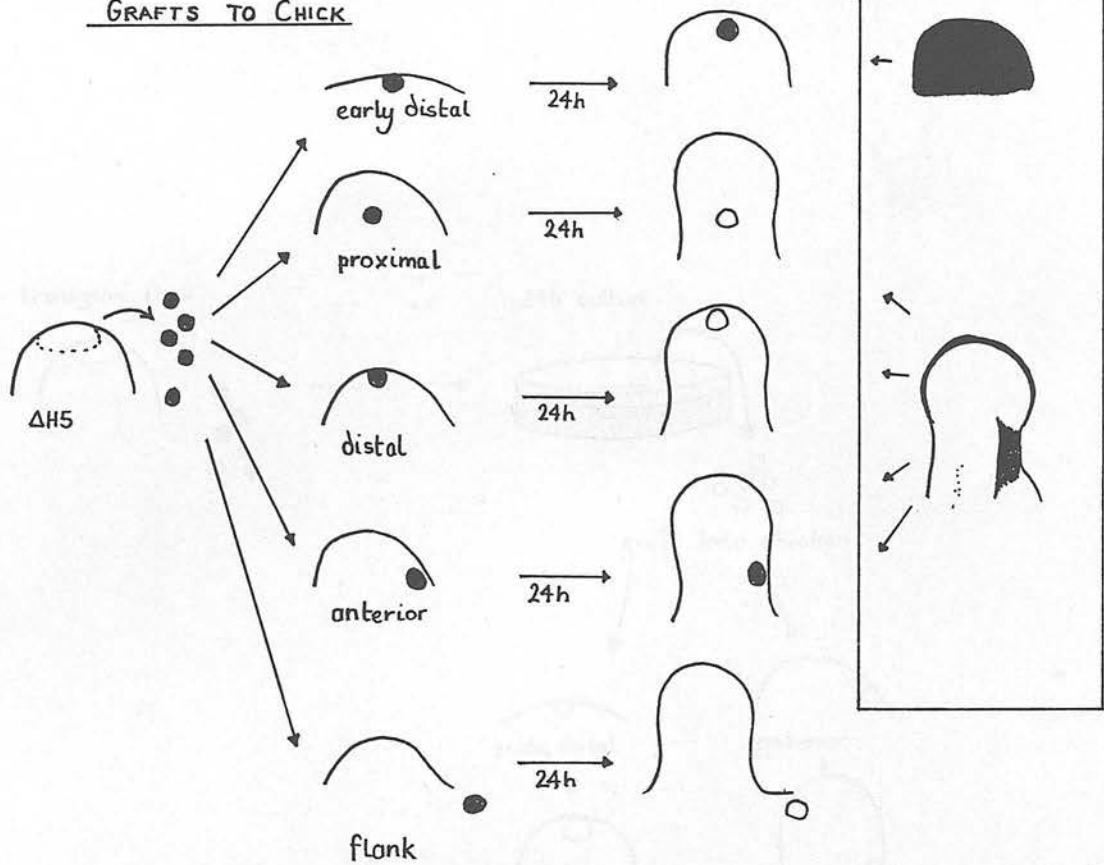
3/3 grafts to the anterior margin of stage 24/25 wing were also negative for β-Gal (Figure 6.11 K) .

This experiment confirmed that W4 Progress Zone mesenchyme which had downregulated the transgene could not subsequently reinitiate it. The experiment was incomplete, had no positive control, and no attempt was made to study expression of the endogenous gene in these conditions. Further work is necessary to confirm the result.

TISSUE CULTURE



GRAFTS TO CHICK



GRAFTS TO MOUSE

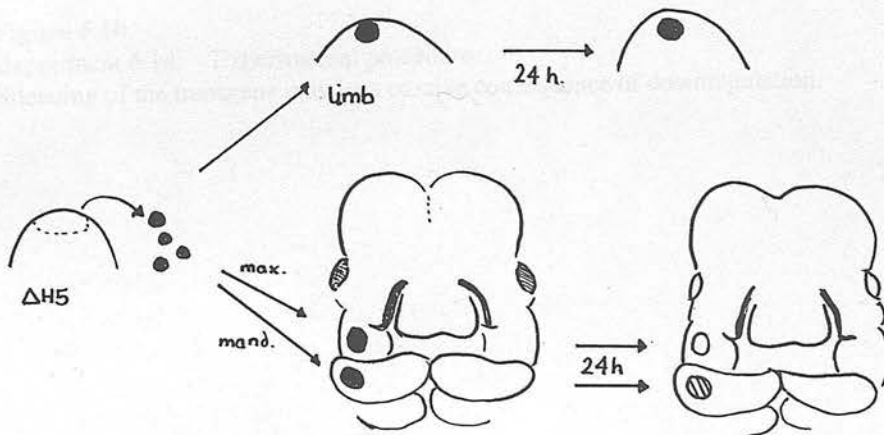


Figure 6.9:

Experiments 6.12 and 6.13

Expression of W3/4 Progress Zone mesenchyme can be maintained for 24 hours by grafting into areas of the chicken or mouse which would be expected to express the transgene.

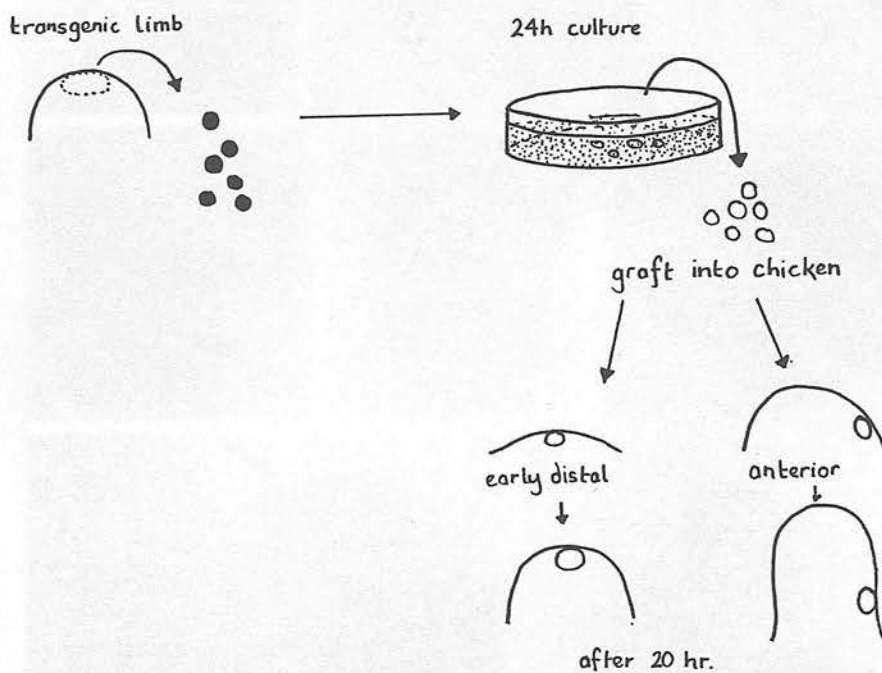


Figure 6.10:

Experiment 6.14. - Experimental procedure.

Silencing of the transgene may be a passive consequence of downregulation.

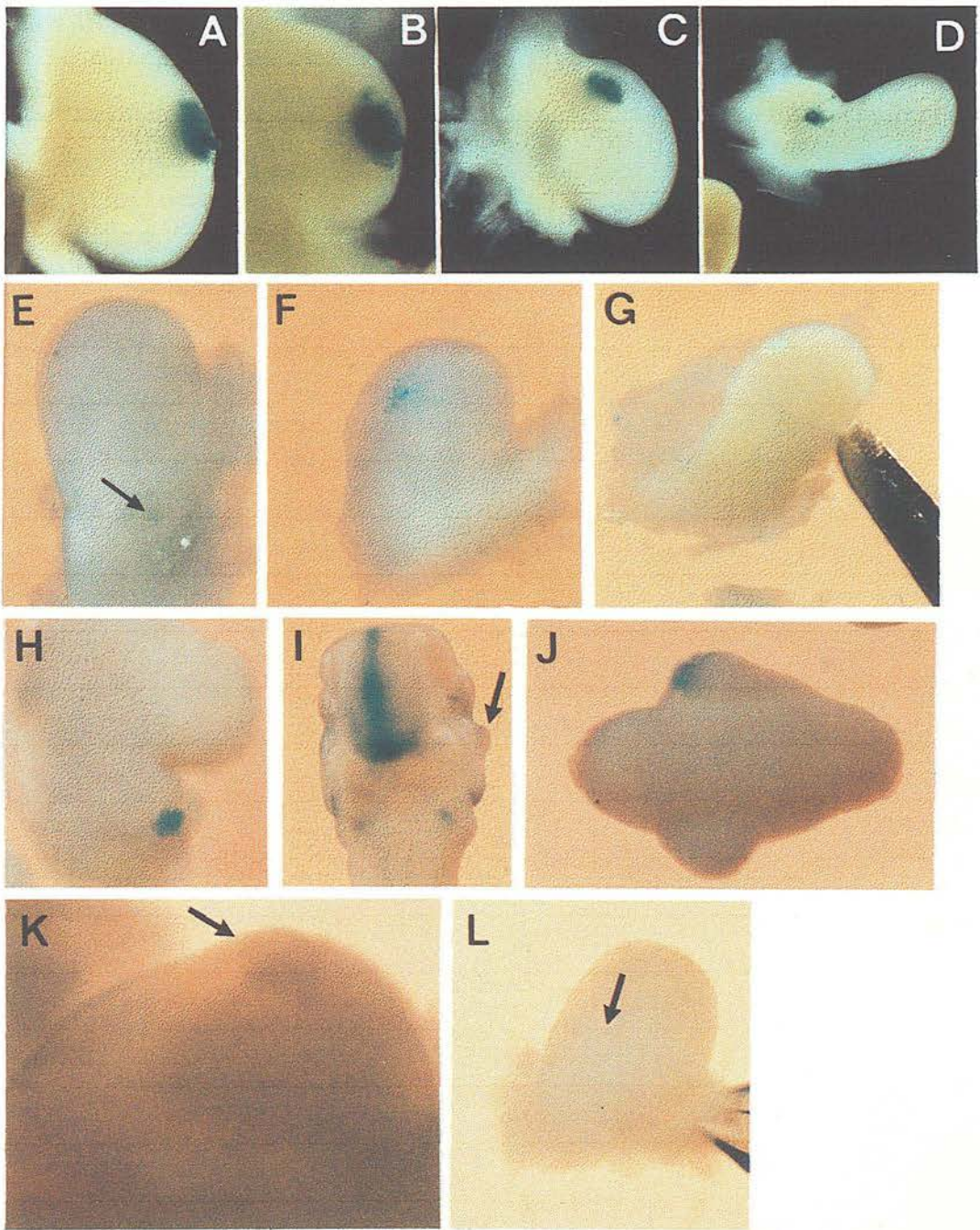


Figure 6.11

Experiments 6.12, 6.13 and 6.14

A-G. Grafts of W3/W4 Progress Zone mesenchyme into chick wings. After 24 hour culture, grafts remain blue only within areas of the wing equivalent to the transgene-expressing domains of the mouse limb.

H, I, J. Grafts to the mouse mandible (H), maxilla (I), or early limb bud (J). Expression of *lacZ* is maintained in the mandible and limb, but not in the maxilla.

K, L. Experiment 6.14. W3 limb mesenchyme cultured for 24 hours *in vitro*, then grafted into early (K) or anterior (L) chick wings does not reinduce expression of *lacZ*.

Overview and conclusions:

The experiments contained in this chapter have indicated that the control of expression of the transgene is not in all respects analogous to control of the endogenous *Msx1*. Experiments with the 10.5 day Progress Zone mesenchyme have shown that the reporter is open to extrinsic signals, and that these signals are the same in mice and chickens, but that the response of the transgene to these signals is not the same as that of *Msx1*. In particular, I have still not shown maintenance of transgene expression by the AER.

E11.5, W6, Progress Zone mesenchyme which is still capable of expressing *Msx1* when grafted into the chicken, is not capable of reinitiating the transgene in several experimental situations. Neither is W4 Progress Zone tissue which has been allowed to downregulate the transgene in culture. There may be negative regulators which bind to the promoter during downregulation and which cannot be overridden subsequently by the action of extrinsic inductive factors. The implications of these results will be discussed in Chapter 8.

Experiment 7.10

I have described transgene expression in a population of cells in the anterior of the limb-bud which I have shown to be differentiating myocytes. The implications of these cells for mechanisms on transgene control will also be discussed (Chapter 8). These cells have uses as markers for myocyte behaviour *in vivo* and suggest a number of future lines of work. Already they have produced the (not trivial) result that mouse myocytes can contribute to chicken muscles, although it is not yet clear whether they can fuse with chicken myotubes. The implications of this aspect of expression for the control and function of the *Msx* genes will also be discussed.

CHAPTER SEVEN

Bone Morphogenetic Protein-4 is an upstream regulator of *Msx* genes in the limb

Introduction:

Having analysed the expression pattern of *Msx* genes in the developing limb buds of mice and chickens (Chapter 3), a correlation became apparent between these genes and the expression of *bone morphogenetic protein-4* (*Bmp4*) at all stages of limb development. BMP4 had previously been shown to be able to induce *Msx1* in cultured dental mesenchyme. I proposed that BMP4 could also induce *Msx* genes in the limb.

Experiment 7.01

Application of exogenous BMP4 to the limb bud causes ectopic expression of *Msx1* and *Msx2*:

Beads, soaked in BMP4, were implanted into proximal central mesenchyme of the stage 22/23 limb bud, behind the Progress Zone, away from the marginal domains of *Msx* expression.

Following a protocol which was an amalgam of those laid down by various workers, heparan acrylic beads were soaked in 10 or 100ng/ml BMP4 before implantation into a cut made in the dorsal surface of the limb. After an overnight culture, the beads were located proximally in stage 25/26/27 limbs in regions which would not be expected to express the *Msx* genes (Figure 7.1).

17 implantations were performed, 10 using 100ng/ml and 5 using 10ng/ml BMP4. 2 beads soaked in PBS were also implanted.

14 chickens survived, the limbs were amputated (the beads carefully removed) and were fixed in 4%PFA. They were dehydrated, cleared and wax embedded as described previously, then sectioned for radioactive *in situ*.

Representative slides from six limbs were hybridised with chicken *Msx1* and *Msx2* probes.

In 6/6 cases, strong expression of both *Msx1* and *Msx2* was detected, specifically around the BMP-soaked beads. (Figure 7.2 A-D)

There was apparently no difference in the level of ectopic expression induced by beads soaked in 10 or 100ng/ml. Ectopic expression was at least as strong as 'normal' expression in the marginal mesenchyme. Intensity of signal dropped with increasing distance from the bead.

There was no *Msx* gene expression around beads which had been soaked in PBS. (1/1).

The effect which implantation had on the fate of cells around the bead was not apparent after overnight culture. Some of the cells around the beads showed swollen, strongly-stained nuclei which was reminiscent of apoptotic cell death.

Conclusion:

That the presence of ectopic BMP4 within the intact developing limb bud can induce ectopic expression of both *Msx1* and *Msx2* within 18 hours.

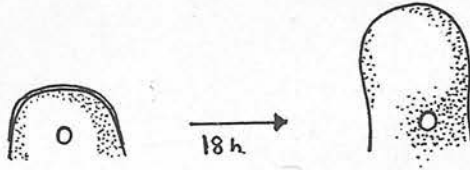


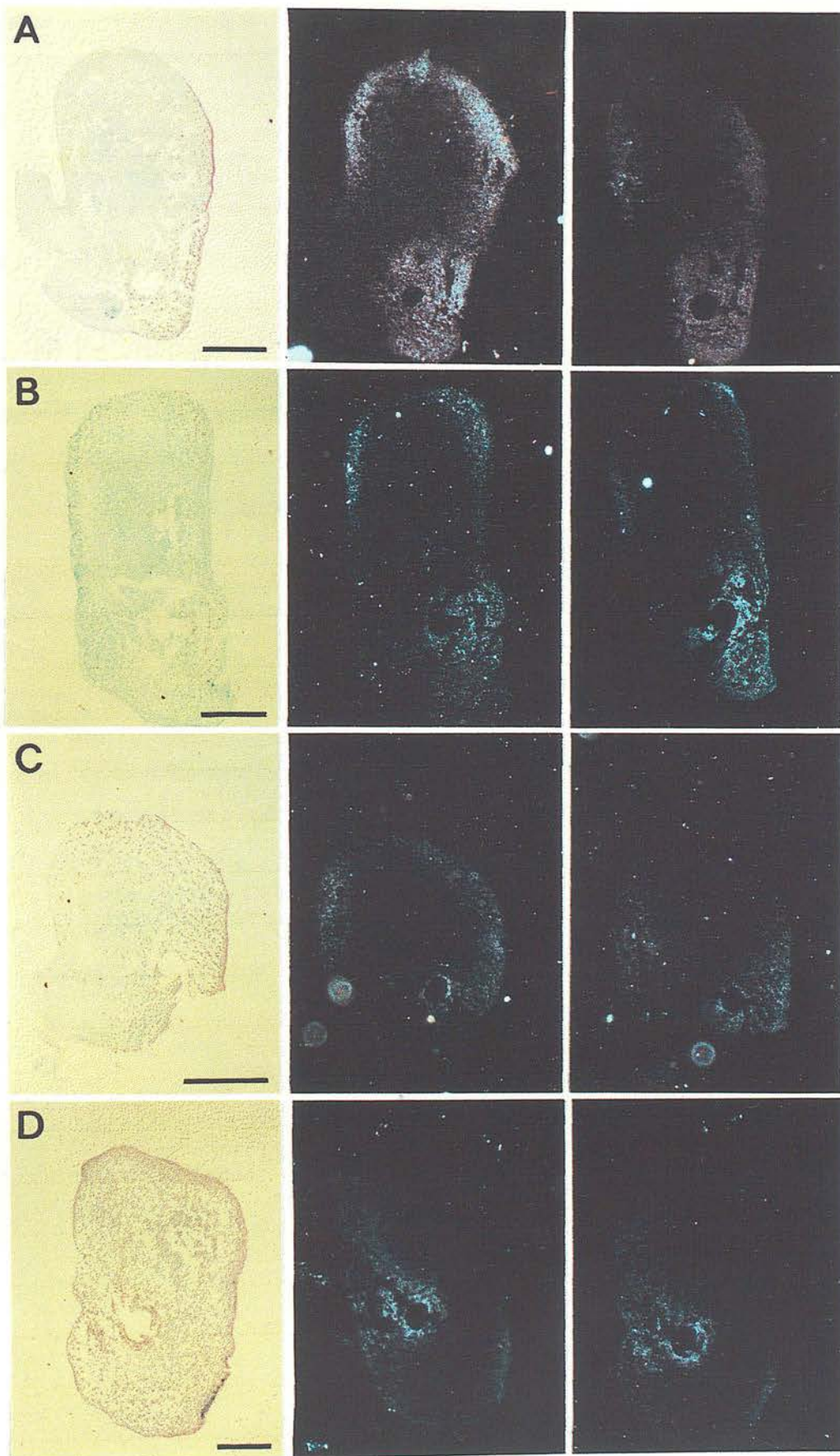
Figure 7.1:

Experiment 7.01 - Implantation of a BMP4-soaked bead into the chick limb induces expression of *Msx1* and *Msx2* in the proximal core of the limb.

Position of bead before and after overnight incubation is shown. Anterior is to the right.

Figure 7.2

BMP4 induces expression of *Msx1* and *Msx2* in the limb. A, B, C are *in situ* results on sections of chicken limbs into which beads soaked in 100ng/ml BMP4 were implanted, followed by fixation after 18 hours *in ovo*. For D, 10ng/ml BMP4 was used.
Left to right in each row = bright-field, *Msx1*, *Msx2* respectively.
Scale bar = 500µm



The effect of BMP4 on expression of the Δ H5 transgene

I decided to investigate whether transgenic Δ H5 tissue could also respond to BMP4.

The two questions which I wanted to ask were whether BMP4 could induce the transgene *de novo* in non-expressing tissue, and whether it could maintain the transgenic activity in expressing tissue.

Experiment 7.02

BMP4 cannot induce transgene activity in the handplate of 11.5d fore-limbs

11.5d Δ H5 fore-limbs (W5) were placed in CMRL1066 medium with 10% FCS. A cut was made in the middle of the digit plate into which beads were inserted. The beads had been soaked in 0, 10 or 100ng/ml BMP4 (2,2 and 4 limbs respectively).

After 18 hours culture, during which time the limb healed around the bead, the limb was fixed and stained. In no case was there any expression of the transgene around the bead (Figure 7.3 A-C).

Experiment 7.03

BMP4 cannot maintain expression of the transgene in E10.5 Progress Zone mesenchyme

10.5d, W3/4, limbs were trypsinised and de-epithelialised, the mesenchyme placed into 1066 medium with 10% FCS. Beads, soaked in 0, 10 or 100ng/ml BMP4 were implanted into the limb mesenchyme. (4, 2 and 5 beads respectively, a total of 11 limbs)

After 24 hours culture, the mesenchyme was fixed and stained.

The results from the different treatments were indistinguishable - the mesenchyme was clear with a large patch of blue cells, which need not be centred on the bead. All limbs were therefore indistinguishable from limbs without beads. (Figure 7.3 D-I)

Conclusions:

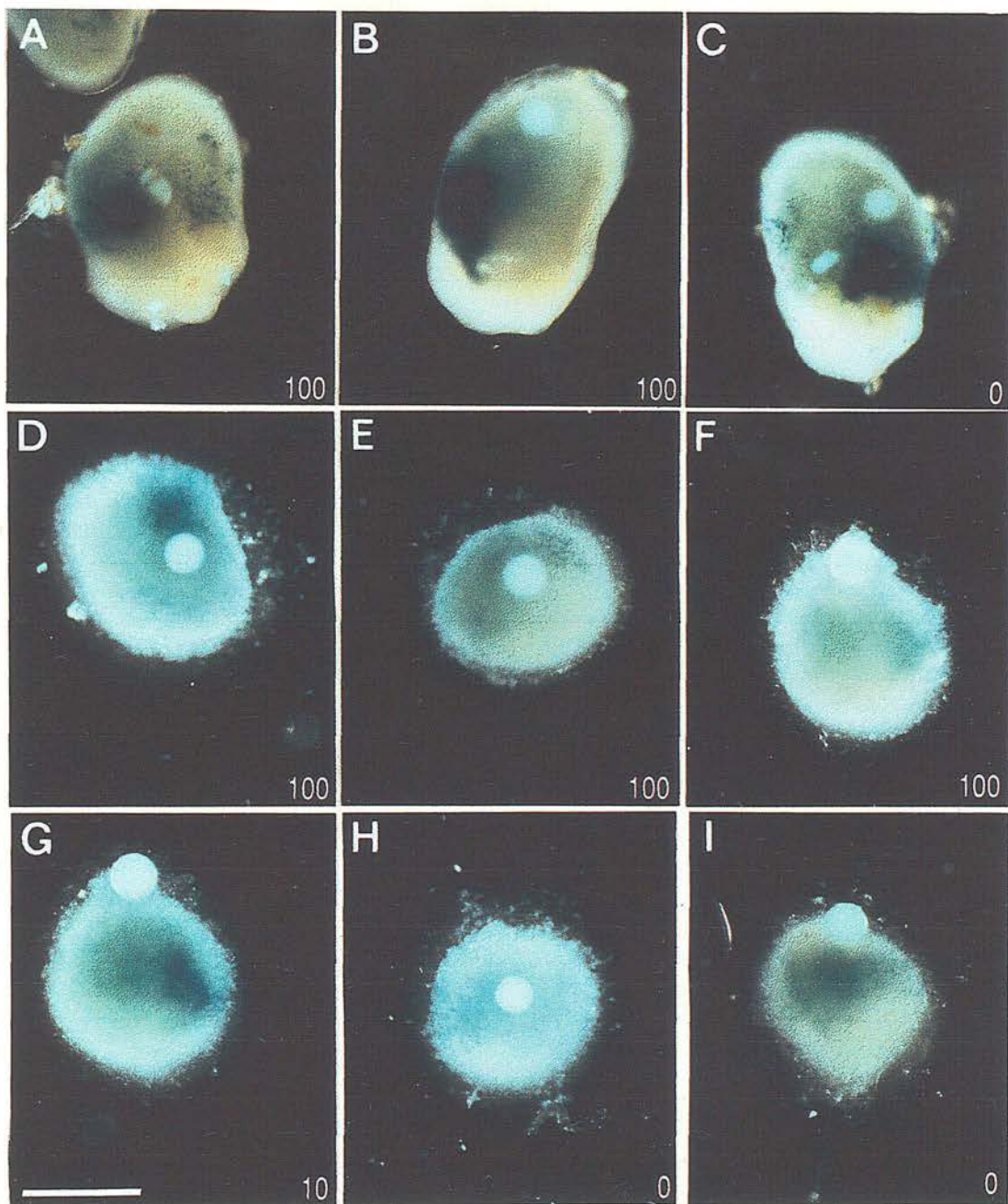
BMP4 cannot induce the transgene *de novo* in non-expressing W5 limb tissue. This is in contrast to its effect on endogenous *Msx1*.

BMP4 cannot, on its own, maintain expression of the transgene in W3 limb mesenchyme.

The implications of these results for the control of the transgene, of *Msx1* and the nature of the AER signal will be treated in Chapter 8.

Figure 7.3

Experiments 7.02 and 7.03.
W5 limbs (A, B, C) or W3/W4 de-epithelialised limb mesenchyme (D-I), cultured overnight after implantation of a BMP4-soaked bead.. The number in the bottom right-hand corner gives the concentration of BMP4 in ng/ml.
The concentration of BMP4 has no effect on the expression of the transgene.
Scale bar = 500µm



CHAPTER EIGHT

DISCUSSION

1. THE EXPRESSION OF *Mx1* AND *Mx2* IN THE LIMB BUD

The potential for functional redundancy between *Mx1* and *Mx2*:

One of the questions which this study addressed was to see whether *Mx2* could compensate for a lack of *Mx1* in the limb, hence accounting for the normal limbs exhibited by the *Mx1* knockout mice. The question makes several assumptions:

- (i) That *Mx1* has a role in the development of the mouse limb;
- (ii) That the limbs are indeed normal, and that there is no subtle developmental phenotype as yet undetected;
- (iii) That the mutant allele was a genuine null, and did not retain any residual activity or expression of a related cell sequence. In the literature, Carter *et al.* (1995) showed that these sequences may retain expression activity;
- (iv) That if *Mx1* is part of one developmental pathway for the patterning of the mouse limb, there is an associated parallel developmental pathway which can compensate if the former is disabled; and
- (v) That *Mx1* and *Mx2* either through identical functions or simply for the later cell fate to lead to the pattern of the limb, and that they can regulate target genes in the same way.

However, when these assumptions, and none of them seems implausible, the question becomes one of whether the expression patterns of *Mx1* and *Mx2* in the developing limb are similar enough for there to be functional redundancy between them.

And, while not identical, the temporal and spatial expression patterns of the two genes are much more similar than was originally thought. The origins of *Mx1* in the Prospective Zone, *Mx2* in the AER (e.g. Akita *et al.* 1993) are, for now, a highly quantifiable statement. *Mx1* is generally more strongly expressed in the ectoderm than *Mx2*, which is more strongly expressed in the ectoderm. This does not necessarily preclude functional redundancy, because the exact of compensatory sequences, but it does mean it is not known.

Nevertheless, the expression differences between the expression pattern of the two *Mx* genes. At the early stages of limb development, in both mice (e.g. 9.5 VZ and 12.5 VZ) and *Ch. trichostepus* (17-18), *Mx1* is

CHAPTER EIGHT

DISCUSSION

1. THE EXPRESSION OF *MSX* GENES IN THE LIMB BUD

The potential for functional redundancy between *Msx1* and *Msx2*:

One of the questions which this study addressed was to ask whether *Msx2* could compensate for a lack of *Msx1* in the limb, hence accounting for the normal limbs exhibited by the *Msx1* knockout mice. The question makes several assumptions:

- (i) That *Msx1* has a role in the development of the mouse limb;
- (ii) That the limbs are indeed normal, and that there is no subtle developmental phenotype as yet undiscovered;
- (iii) That the mutant allele was a genuine null, and did not retain any residual activity on account of it retaining all sequences 5' to the homeobox - Catron *et al.* (1995) showed that these sequences may retain repressor activity;
- (iv) That if *Msx1* is part of one developmental pathway for the patterning of the mouse limb, there is no second, parallel, developmental pathway which can compensate if the former is knocked out.
- (v) That *Msx1* and *Msx2* retain enough sequence similarity for the latter to be able to bind to the targets of the former, and that they can regulate target genes in the same way.

However, given these assumptions, and none of them seems implausible, the question becomes one of whether the expression patterns of *Msx1* and *Msx2* in the developing limb are similar enough for there to be functional redundancy between them.

And, whilst not identical, the temporal and spatial expression patterns of the two genes are much more similar than was originally thought. The dogma of '*Msx1* in the Progress Zone, *Msx2* in the AER' (e.g. Akimanto *et al.* 1995) has become a highly qualifiable statement. *Msx1* is generally more strongly expressed in the mesenchyme than *Msx2*, which is more strongly expressed in the ectoderm. This need not necessarily preclude functional redundancy, because the threshold concentrations necessary for protein function are not known.

Nevertheless, there are differences between the expression patterns of the two *Msx* genes. At the early stages of limb development, in both mice (stages W1-W2) and chickens (stages 17-18), *Msx2* is

restricted to the anterior two-fifths of the mesenchyme, but *Msx1* is more broadly expressed (Compare Figs. 3.1A,B; 3.6A,B,C with Figs. 3.2A,B; 3.7A,B,C). If *Msx1* has a role in early limb development, such as induction of the AER, and if there is to be functional redundancy between the genes, then it is necessary to believe that either it is only anterior expression which is important, or there is enough *Msx2* in the mesenchyme of the knockout mice to fulfil this role.

In chickens, no *Msx2* was detected in the postaxial distal mesenchyme before stage 26. For chickens, in contrast to the mouse, *Msx1* expression fades out completely in the posterior Progress Zone before reinitiation in the digit plate associated with cell death (as if the chicken carries to completion a distal downregulation, a shadow of which is seen in the W4 mouse) (Compare Fig. 3.1E with Fig. 3.6H). Therefore, in respect of the lack of distal expression of *Msx2* in the chicken, it seems that the gene merely anticipates the expression pattern which will be shown by *Msx1*.

Msx1 is more extensively expressed in the interdigital zones of the autopod than *Msx2* (which was only detected in the apoptotic zones, and not strongly in the prospective soft tissue surrounding the chondrifications) (Compare Fig. 3.3E with Fig. 3.4E and see Fig 3.10). I wonder how carefully the soft tissue of the handplate in the *Msx1* knockout mice has been inspected for abnormal tissue patterning.

The other major differences between the expression of *Msx1* and *Msx2* are in regions where *Msx2* is expressed and *Msx1* is not. From the earliest stages of limb outgrowth, *Msx2* is expressed more strongly in the ectoderm, culminating in the strong and widespread expression in the AER, only a shadow of which is seen for *Msx1*. If mice which were homozygous null for *Msx2* had limb truncations, then a failure to maintain the Progress Zone by the ridge due to lack of *Msx2* function would be implied; however, *Msx1* is expressed in the ridge in detectable amounts, especially during early stages, so there may be the potential for some functional redundancy between the two genes in the ridge also.

The expression of *Msx2* around the chondrifying carpals in the digit plate of the W7 and W8 mouse limb, and subsequently in the preaxial fatty pad tissue of the fore-limbs, areas which do not express *Msx1*, is an example where functional redundancy would not be possible, and if *Msx2* has a necessary function here, it would be a potential site for a mutant phenotype in an *Msx2* knockout mouse.

It is not certain that one should expect the *Msx1* and *Msx2* proteins to be completely functionally interchangeable. The experiments of Song *et al.* (1992) showed that ectopic expression of *Msx1*, but not *Msx2* could prevent differentiation in F3 pre-myogenic cells. They have also shown (Wang and Sassoon, 1995) that *Msx1* downregulation precedes expression of MyoD in cultured mouse limb mesenchyme cells. Davidson (1995) has shown that there are sequence domains in *Msx1* protein

which are evolutionarily conserved across species, and sequence domains in *Msx2* which are also conserved, but that the domains are not all conserved between *Msx1* and *Msx2*. This evidence is consistent with *Msx1* and *Msx2* having different, evolutionarily conserved, activities.

It may be that *Msx1* and *Msx2* do not show complete functional redundancy, but that the phenotype is not a primary morphological defect. My interpretation of the experiments of Reginelli *et al.* (1995) (page 56) is that regeneration may occur when cells at the cut stump express one of the *Msx* genes, not necessarily both. In the *Msx1*-knockout mouse, therefore, the testable prediction is that distal tip regeneration will occur only in embryonic handplate when the very distal cells, expressing *Msx2*, are cut through. It implies that although the *Msx1* knockout mice do not show an overt structural defect, the regenerative potential of the limb has been affected.

Correlation of *Msx* gene expression with developmental processes known to be occurring in the limb:

General conclusions:

At all stages of limb development, there is no restriction of *Msx* gene expression to any one tissue type - the expression domains of *Msx* genes encompass areas of prospective cartilage, muscle, connective tissue and epithelium. Transcripts are detectable, apparently uniformly, in every cell within the expression domains. It is possible therefore that the genes are involved in pattern formation in the limb, defining domains in which developmental decisions are being made.

Separable domains of *Msx* expression in the limb, and possible roles for the genes:

By combining what is known about the expression pattern of the *Msx* genes in the limb with the wealth of information about its development (such as the experimental mapping of the chick limb alluded to earlier), we can divide the expression patterns of the genes into domains which may have some functional significance. Conclusions reached on the basis of chicken data may be extrapolated to make predictions about mouse development.

'Early' expression - prior to ridge formation:

The early expression of the *Msx* genes may be a consequence of their expression in the somatopleure in areas which include the limb field. *Msx1* and *Msx2* are expressed in different, but overlapping mediolateral domains in both the ectoderm and the underlying mesoderm of the limb field at 8-8.5 days, as part of a larger domain of expression which runs down the anterior/posterior axis of the embryo from the level of the front of the fore-limb, backwards (Davidson and Hill, 1992). As a consequence of these differing mediolateral extents of expression, when the body wall folds round at E8.5-9 to form a recognisable embryo shape, *Msx2* comes to lie more ventrally in the limb field than *Msx1*.

That these expression patterns remain intact up to ridge formation may indicate that the genes are expressed in the early limb bud as a cell lineage effect, or under control of the same signals which initiated expression in the gastrula.

The AER-Progress Zone relationship:

The experiments of Davidson *et al.* (1991), Coelho *et al.* (1993), Robert *et al.* (1991), as well as studies of *talpid*³, *limbless* and *diplopodia-5* mutants have established that the two-way interaction between the apical ectoderm and the distal mesenchyme (Amprino, 1965) can affect the transcription of the two *Msx* genes; the function of the genes during this interaction is, as yet, uncertain.

The diagrams in Milaire (1965) describe cytochemically (on the basis of increased RNA content in the basal cytoplasmic columns of cells and higher cytoplasmic levels of dephosphorylases) the thickened areas of ectoderm which are associated with maintenance of proliferation in the mesenchyme by this two-way interaction. These domains are very like *Msx* expression patterns. Taken together, these lines of evidence link upregulation of the *Msx* genes with the epithelial-mesenchymal interaction taking place distally in the mouse limb.

It remains possible that *Msx* gene expression in the early Progress Zone may inhibit differentiation in these cells (Wang and Sassoon, 1991; Song *et al.*, 1992), but the lack of *Msx* gene expression in much of the Progress Zone of the chicken between stages 23 and 26/27, (when ridge-removal experiments have shown the classically-defined Progress Zone to be still active), argues that this is not necessarily the case.

The anterior mesenchymal expression of Msx1 and Msx2:

Two lines of evidence from chicken have shown that the control of expression in the mesoderm along the anterior boundary of the limb is qualitatively different from that in the Progress Zone. Firstly, removal of the AER from a stage 20 chick limb results in almost immediate cessation of *Msx1* expression in the posterior distal quarter of the limb, but the anterior domain of expression (including cells in the anterior Progress Zone) remains active for at least 24 hours (Ros *et al.*, 1992). Secondly, whereas *in vitro* experiments have shown that the AER can induce expression of *Msx1* strongly and *Msx2* only weakly in cultured mesenchyme upon which it is overlaid, anterior non-ridge ectoderm from the stage 24 limb can induce both genes strongly in underlying mesenchyme (Coelho *et al.*, 1993a)

The anterior mesenchymal cells of the limb are non-chondrogenic and developmentally plastic (Anderson *et al.*, 1994), in that their fate can be respecified by simple experimental manipulation, such as the implantation of a bead soaked in retinoic acid (Tickle *et al.*, 1982). The *Msx* genes may be involved in the maintenance of this developmental plasticity. For example, the domain of cells in the stage 21 chick limb which are competent to respond to ectopic expression of *FGF2* (introduced on a retroviral vector) and produce duplications (Riley *et al.*, 1993) is coincident with the domain of *Msx1* expression as revealed by the present study (pers. obs.).

Msx gene expression may be involved with specifying the 'anteriorness' of these cells. Application of the posteriorising molecule, retinoic acid, has been reported to cause downregulation of *Msx* gene expression in the anterior of the limb (although this is *contra* Wang and Sassoon, 1995); in mutants such as *talpid*³ and *diplopodia*⁵ in which, by a number of genetic and morphological markers, the a/p axis has been shown to be disrupted, the anterior domain of *Msx2* and possibly *Msx1* is lost (Coelho *et al.*, 1993c; Krabbenhoft and Fallon, 1993).

The interdigital necrotic zones (INZ):

Msx gene expression is reinitiated *de novo* in mesenchymal cells around the margin of the autopod and interdigitally, in both chickens and mice, coincident with the start of cell death in these zones and continuing as the episode of cell death progresses. When comparing the expression pattern of the homologous genes in mice and chickens, it is the distal fade-out of *Msx1* expression at stage 24 of the chicken which is the major difference between the behaviour of this gene in mouse and chicken limbs of an equivalent stage. Chicken *Msx1* reinitiates distally at stage 27/28. Mouse *Msx1* expression never completely fades out distally, although it is quite weak in the posterior distal region at stage W4. It may be that *Msx1* has done its jobs in the Progress Zone by this stage, and that the marginal autopodial expression in the W5 and W6 mouse limbs may be (compared to chicken) a relatively

early reinitiation of the gene. Thus photographs of stage W6 limbs in previous publications (e.g. Robert *et al.*, 1989), showing expression of *Msx1* in the 'Progress Zone' round the margin of the handplate, may in fact show expression of *Msx1* associated with initiation of autopodial cell death. *Msx* expression is seen in regions between the digital condensations, where cells are dying. The boundary between *Msx*-expressing cells and non-expressing is not absolutely sharp and does not correspond to any morphological boundary (such as the incipient chondrifications). In common with other areas of *Msx* expression therefore, I suggest that the *Msx* expressing regions specify developmental domains, as yet undefined, rather than being associated with any specific cell type.

***Msx* genes and cell death pathways:**

Expression of *Msx2* dorsally in rhombomeres 3 and 5 of the mouse correlates with apoptotic elimination of prospective neural crest cells. There is evidence that *Msx* expression in limb mesenchyme may be associated with apoptotic cell death in mice and chickens. The chicken anterior, posterior and interdigital necrotic zones lie neatly within the domains of *Msx1* and *Msx2* expression (Compare Figs. 3.6 and 3.7 with Fig. 1.6). Mice also have interdigital and anterior necrotic zones within *Msx* expression domains.

I would argue, however, that *Msx* expression is not mechanically linked to the process of cell death. In all cases the areas of cell death are smaller than the *Msx* expression domains as if the *Msx* genes are marking out a region of cells, a *subset* of which are destined to die. The data suggests that *Msx2* may be more closely related to cell death than *Msx1*, since *Msx2* mesenchymal expression is more tightly restricted to the apoptotic zones. This may be a genuine result, or it may be a consequence of *Msx2* transcripts being generally less abundant in the mesenchyme.

I found that *Msx* gene expression precedes the start of cell death, at least in the chicken PNZ, where *Msx* expression starts at the stage when cells are irreversibly committed to death (as defined by Saunders Jnr., *et al.* (1962)). Expression of both *Msx1* and *Msx2* is maintained in the PNZ at stage 26, when cell death stops briefly (Saunders Jnr. *et al.*, 1962).

In the interdigital zones the *Msx* genes may be downregulated before cell death is complete and there is no *Msx* expression in other areas of cell death, such as the 'opaque zone' which separates the radius and ulna (tibia/fibula).

There is therefore no one-to-one relationship between *Msx* expression and cell death. The domains of *Msx* expression may however mark groups of cells which may be receptive to cell death signals. In the *limbless* chicken mutant, the failure of the AER in the fore-limb results in cell death throughout the Progress Zone (Robert *et al.*, 1991); in the hind-limb of *wingless* chickens (in Hinchliffe and

Johnson, 1980), the anterior necrotic zone apparently expands to fill the whole domain of anterior *Msx2* expression

***Msx* genes and the differentiation process:**

There are cases where expression of *Msx* genes is definitely not associated with cell death. In the Progress Zone, *Msx1* expression is associated with the *survival* of cells in an undifferentiated, highly proliferative state (Wang and Sassoon, 1991) and *loss* of *Msx1* expression in the posterior Progress Zone precedes cell death (Ros *et al.*, 1992). In the perichondrium, *Msx* genes are expressed in cells which can differentiate into other cell types, e.g. osteoblasts.

The association of *Msx* gene expression at sites of mesenchymal-epithelial interactions throughout the body is well known; in the teeth (Jowett *et al.*, 1993), the membranous bones of the skull (Takahashi *et al.*, 1991), and the female reproductive tract (Pavlova *et al.*, 1994), *Msx* expression has been shown to be correlated with the correct differentiation of tissues in these structures. *The Msx genes may be involved in marking cells as having the ability to respond to extraneous signals (such as those produced by epithelial-mesenchymal interactions) by making a developmental decision, whether that decision be differentiation, proliferation or death.*

It is perhaps a little easy to say that 'expression of *Msx* genes marks cells which retain the ability to make a developmental decision' without thinking about the implications of this, or at least considering what a developmental decision really is. If this is truly what the *Msx* genes are doing, it is implied that the actual nature of the cells' developmental decisions is controlled at another level. In order to be pushed into a canalised fate pathway by tissue interactions, cells must both receive extrinsic signals and be competent to respond to them. *Msx* genes may provide the competence, and the extrinsic signals (working in concert with intrinsic factors such as gene activity initiated by *Hox* gene expression) impose the nature of the response.

At points in the developmental pathways which create the limb, certain sets of cells will be expressing one or both *Msx* genes, and others will not. Expression of *Msx* genes, as transcription factors, potentially changes the expression of a number of downstream genes. To describe what these downstream genes might be is to attempt to catalogue an infinite number of unknowns! They may be cell surface receptors or members of signal transduction pathways. They may equally be unconnected with signal transduction pathways - the downstream genes may cause physiological changes in the nucleus or cytoplasm which alter the cell's capacity to produce the structural proteins which potentiate morphological differentiation and cell movement. It is likely that *Msx1* and *Msx2* have

different activities, but it is not known how different - it is unlikely that they have identical effects on the activity of downstream genes (page 206).

Whatever the mechanism of downstream gene action, expression of the *Msx* genes may change the kinetics of the cascade of gene interactions within the nucleus. Seen in this light, as basic cogs in the machine, there is little difference between the actions of the *Msx* genes and those of the *Antp*-class homeotic genes (page 7). The inadequacies of this treatment of the genes will be brought to the fore when there is a better knowledge of the genetic pathways within which the *Msx* genes act. There is obviously a pressing need for a direct investigation of genes which may be upstream or downstream of *Msx1* and *Msx2*.

***Msx* and other genes:**

It is of course useful to investigate which genes are coexpressed with *Msx* genes and which may therefore be involved in the same developmental pathways. One possible interaction in which *Msx* genes could be involved is the one whereby the posterior AER, expressing *Fgf4* (Niswander and Martin, 1992), prevents cell death in the underlying AER and also maintains the ZPA (Vogel and Tickle, 1993), expressing *Bmp2* (Francis *et al.*, 1994) and *Sonic hedgehog* (Riddle *et al.*, 1993). The ZPA imposes positional values across the anterior/posterior axis of the limb and, in turn, appears to maintain proliferation in the Progress Zone. However, correspondence of the expression of *Msx* genes with the expression of the genes involved in this interaction is not tight. *Msx2* and, initially, *Msx1* are expressed throughout the ridge, not just posteriorly like *Fgf4*; and, whereas the posterior mesenchymal domain of *Msx* expression does partially encompass the ZPA, polarising activity in this region is found before *Msx* expression.

Many other developmental genes are expressed in the limb in domains which partially overlap with the domains of the *Msx* genes. Even the briefest *resume* includes *Wnt5a* (ventral ectoderm, ridge, distal mesenchyme, interdigitally) (Gavin *et al.*, 1990; Parr *et al.*, 1993), *En1* (ventral ectoderm) (Davidson *et al.*, 1988), *Evx1* and *Evx2* (distal mesenchyme) (Dolle *et al.*, 1994), all four *Hox* clusters (various different anterior/posterior and proximo/distal restricted domains) (Duboule, 1994; Izpisua-Belmonte *et al.*, 1992; Yokouchi *et al.*, 1992; Peterson *et al.*, 1994; Charite *et al.*, 1994), the retinoic acid receptors (disparate expression patterns, including the necrotic zones) (Mendelsohn *et al.*, 1992), *Fgf2* (ridge and throughout the mesenchyme, strongest distally) (Savage *et al.*, 1993), *Prx1* (Nohno *et al.*, 1993), *Cx43* (Yancey *et al.*, 1992; Pusek *et al.*, 1992; Green *et al.*, 1994), *LEF1* (Oosterwegel *et al.*, 1993), *Dlx1* (Dolle *et al.*, 1992), AV1 (Ohsugi and Ide, 1993) etc. Unfortunately, the role of these

genes in limb development has not been satisfactorily defined either. *Wnt5a* has already been implicated as regulating *Msx1* in the uterine epithelium (Pavlova *et al.*, 1994). *LEF1* is associated with sites of mesenchymal-epithelial interaction throughout the embryo (Van Genderan *et al.*, 1994); *Cx43* is certainly involved in facilitating cell-cell interaction during embryonic development, and knockout mice show heart defects (Reaume *et al.*, 1995) which may mirror those of the *Msx2* mutant mice. These three genes, at least, are strong candidate players in a genetic pathway which includes the *Msx* genes.

Data from Grindley *et al.*, (1995) suggests that *Pax6* may be upstream of *Msx1* in the nasal placodes. This raises the possibility that *Pax3* is upstream of *Msx1* in the ventrolateral somites and migrating myocytes.

The relationship between Fgf2-responsiveness (Riley *et al.*, 1993) and the domain of *Msx1* expression has been mentioned previously. Fgf2 has furthermore been shown to maintain *Msx1* expression in the Progress Zone (Fallon *et al.*, 1994) and in cultured limb cells (Watanabe and Ide, 1993).

The first result to come from analysis described in Chapter 3 was the possibility of an interaction between BMP4 and the *Msx* genes. Anyone who compares the expression pattern of *Bmp4* in the limb (Francis *et al.*, 1994; Jones *et al.*, 1991) with the expression of the *Msx* genes as shown in this analysis, cannot fail to be struck by the similarity. *Bmp4* has also been shown to be closely co-expressed with *Msx2* in the hindbrain (Graham *et al.*, 1994) and the developing tooth (Heikenheimo *et al.*, 1994; Vaino *et al.*, 1993; MacKenzie *et al.*, 1991, 1992), and can act as an inducer of *Msx1* and *Msx2* in cultured tissue. *Bmp4* expression in the tooth appears to be a marker for the ability to respond to signals passing between epithelium and mesenchyme; thus in terms of function, as well as expression pattern, *Bmp4* expression seems to be relevant to the processes in which the *Msx* genes are implicated.

If the apparently anomalous phenotype of the *Msx1* knockout mice is to be unequivocally explained, and if we are to know the function of *Msx* genes in the limb, it may be worthwhile to approach the problem from the *Bmp4* angle.

2. THE FUNCTIONAL SIGNIFICANCE OF AN INTERACTION BETWEEN BMP4 AND MSX GENES

Bmp4 is an upstream activating molecule for *Msx* genes in the limb

I showed that the implantation of a bead, soaked in BMP4, into the proximal limb mesenchyme causes ectopic expression of both *Msx1* and *Msx2* around the bead within 18 hours or less. Because of:

- (i) the low concentration of BMP4 which produces a response,
- (ii) the induction of *Msx1* and *Msx2* by BMP4 in dental mesenchyme and rhombomeres (both *in vitro*),
- (iii) the close correlation between *Msx* and *Bmp4* expression patterns in the limb,

I conclude that BMP4 is an upstream activator of *Msx1* and *Msx2* in the limb.

The bone morphogenetic proteins:

Demineralised bone matrix, when implanted subepidermally or intramuscularly in adult vertebrates, can induce ectopic bone formation by the endochondrial pathway (Reddi and Huggins, 1972). Some osteosarcomal cell lines have the same effect. The active ingredients in these implants, any one of which can induce bone formation, are the bone morphogenetic proteins, BMP1 to BMP8 (Sampath and Reddi, 1981; reviewed in Rosen and Thies, 1992)

BMPs2-8 are sequence-related; they are members of the larger family of TGF β related molecules (Kingsley *et al.*, 1992, review) (although the BMPs are more closely related to each other than they are to other family members). They are diffusible, secreted, cell-signalling molecules which bind as homo- or heterodimers to cell surface receptors. Known receptors, serine-threonine kinases, have been divided on the basis of sequence similarities into two types, typeI and typeII, which act cooperatively to initiate the BMP-response (Hogan, 1994; Letsou *et al.*, 1995; Ruberte *et al.*, 1995).

Different bone morphogenetic proteins are active at different sites and different stages during endochondrial bone formation (Erlebacher *et al.*, 1995). *Bmp5*, defects in which are responsible for the mouse *short ear* mutation (Kingsley 1994 review), is expressed at the earliest condensation of many cartilage elements, which fail to form in the mutant mice. *Bmp3* is expressed in the periosteum and *Bmp6* in hypertrophic cartilage (Kingsley, 1994).

Whatever their *in vivo* role, the application of any single Bmp is enough to initiate the whole bone-forming gene cascade. BMP2 can turn on cartilage specific genes in condensed cells from

immortalised limb cell lines, then can induce expression of bone-specific genes in chondrogenic cells (downregulating the cartilage-specific genes) (Rosen *et al.*, 1991,1994).

The BMPs are implicated as mediators of the cell-cell signalling which occurs during bone formation.

BMP4:

BMP4 is 94% identical at the amino acid level to BMP2; they are the vertebrate homologues of the *Drosophila decapentaplegic* gene, *dpp* (Rosen and Thies, 1992; Wozney, 1992). *Bmp4* may be expressed during embryonic bone formation, but this is not proven. It is expressed in bone repair after breakage (which occurs by the endochondrial pathway) - it is detected in the proliferating periosteum, the medullary cavity and in muscles near the fracture site during repair (Nakase *et al.*, 1994) i.e. like *Msx* genes, it correlates with developmental plasticity and the re-differentiation process.

If BMP4 is added to cultured limb mesenchyme from a stage 24 chicken, cells chondrify as a sheet (Chen *et al.*, 1991) and also express some bone marker proteins. The effect is a direct one on the cells, rather than a passive consequence of withdrawal from the cell cycle, and can be inhibited by PDGF (Chen *et al.*, 1992). BMP2 and BMP3 have the same effect (Wozney, 1992; Carrington *et al.*, 1991).

The chondrogenic effect of BMP4 is a paradox, for BMP4 is, with *Msx* genes, expressed in the marginal, non-chondrogenic mesenchyme of the limb. I can think of two possible explanations for this:

- (i) Condensed mesenchymal cells have been shown to require a signal from the surrounding limb mesenchyme in order to chondrify (Solursh *et al.*, 1982) - this signal could be BMP4;
- (ii) Expression of *Msx* genes has been shown to correlate with the process of making a developmental decision (this thesis). The nature of the developmental decision is different in different areas of the limb and is probably determined by signals from surrounding cells (e.g. the epithelium). Adding BMP4 to limb cells in culture may induce *Msx* genes and force a developmental decision. In the absence of any other extrinsic signals, the default decision may lead cells to chondrification.

Hence, a knowledge of the BMP4/*Msx* interaction can help to explain the apparent paradox.

BMPs are expressed at many sites in the developing embryo:

The above also emphasises the fact that BMPs are signals - they need not be bone-specific. BMPs are components of a signalling pathway which is evolutionarily conserved, and of which only the outcomes are changed. *dpp* does not cause bone formation by the endochondrial pathway in flies (it is a dorsalisating factor at gastrulation, in contrast to BMP4 which is a mesoderm ventraliser in *Xenopus*) (Hogan 1994; Jones *et al.*, 1992; Fainsod *et al.*, 1994); *dpp* is also expressed in ectoderm to induce visceral and cardiac mesoderm formation - it upregulates *tinman* (Frasch, 1995).

There has been conservation of the signalling pathways in which the genes are involved - *dpp* protein will cause bone formation if implanted under vertebrate skin (Kingsley, 1994 review) and ectopic BMP4 can rescue a *dpp* null mutant fly (Reddi, 1994 review).

BMP4 has been shown to cause neuronal differentiation in the rat PC12 cell line (Paralkar *et al.*, 1992). The protein (in common with activinA) binds components of the extracellular matrix such as collagenIV and fibronectin - the authors suggested that BMP4 may show only limited diffusion *in vivo*, because of the matrix attachment, and that it may have a role in the growth and repair of nervous tissues.

BMPs are known to be expressed at many sites in the embryo, in differentiating cells with a variety of fates; many are sites of epithelial-mesenchymal interaction (Rosen and Thies, 1992). I suggest that the developmental processes which BMPs regulate may in part result from up- or down-regulation of the expression of *Msx* genes.

Bmp4 is expressed in the limb, but also in mesenchyme and epithelium of the facial processes, in the ventral posterior mesenchyme after gastrulation, in the ventral neuroepithelium of the diencephalon (associated with the developing Rathke's pouch and pituitary), the myocardium, the epithelium of the nasal pits, during tooth development, in r3 and r5 and in the eye (Chan-Thomas *et al.*, 1993; Jones *et al.*, 1991; Vainio *et al.*, 1993; Graham *et al.*, 1994). There is a large degree of coexpression with *Msx* genes, but there are sites which express *Msx* genes where BMP4 is not found.

Other members of the TGF β family are partly co-expressed with *Msx* genes. *Bmp2* is expressed in the heart, the ZPA, the whisker follicles, dorsally in the diencephalon and in the otic vesicle epithelium (Chan-Thomas *et al.*, 1993; Francis *et al.*, 1994; Lyons *et al.*, 1995). Expression of Tgf β itself is detected at diverse sites during embryogenesis, including facial mesenchyme, nasal sinuses, meninges, teeth, hair follicles and heart valves (Heine *et al.*, 1987). Whereas *Bmp4* is not expressed in the endocardial cushions, *Bmp6* is. Given the similarity of the TGF β family members, the potency

of BMP heterodimers (Lyons *et al.*, 1995) and the undoubted promiscuity of BMP receptors (Letsou *et al.*, 1995; Smith 1995) (below), it is possible that different TGF β -related molecules could act as upstream regulatory factors to *Msx* genes in different areas of the body, to different degrees.

Promiscuity of receptors for molecules in the TGF β family:

A common receptor for BMP2 and BMP4 has been identified (Koenig *et al.*, 1994; Mishina *et al.*, 1995). This ubiquitously expressed type I receptor, BMP receptor kinase-1, BRK1, was isolated from NIH3T3 cells. It is bound specifically by BMP4 and BMP2, not by TGF β or activin, and forms a complex with the mammalian homologue of the *C.elegans* type II receptor, DAF4 (Estevez *et al.*, 1993). There may be other receptors which bind BMP2 or BMP4, not both, but to some extent these two BMPs are inevitably acting in the same genetic pathways and BMP2 is therefore also a potential activator of *Msx* genes. ten Dijke *et al.* (1994) isolated two type I receptors, ALK3 and ALK6 (activin receptor-like kinase) which bind BMP4. Both of these receptors bind BMP7 also, although a third receptor, ALK2, binds BMP7 and activin, but not BMP4. They succinctly summarised the evidence for promiscuity of binding, but their simplified diagram (Copied in Figure 8.1) ignored the different affinities of different ligands for different receptors.

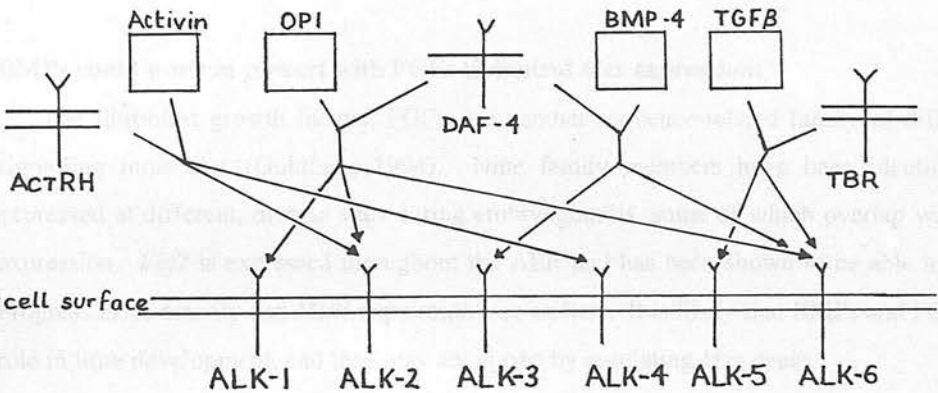


Figure 8.1: (from ten Dijke *et al.*, 1994)

The binding of Activin, BMP7 (OPI), BMP4 and TGF β to the receptors ALK1-6, cooperatively with Activin-receptor (ActRH), TGF β -receptor (TBR) and DAF-4.

BMP4 and genetic pathways:

Little else is known about the genetic pathways in which *Bmp4* is involved, apart from its interaction with *Msx* genes and its effect on dorsal and ventral-specific genes in *Xenopus* mesoderm at gastrulation.

In the limb, BMP2 can be shown to upregulate *Hoxd13*, and is itself upregulated by *Shh*. (Reddi, 1994; Laufer *et al.*, 1994b).

In *Drosophila*, *dpp* has been shown, in different situations, to be upstream of *tinman* and *labial*, but downstream of *Ubx* (reviewed in Andrew and Scott, 1992; Wall and Hogan, 1994). It is expressed in the midgut viscera, where it is required for formation of the gastric caeca and the second constriction. In the anterior midgut, it represses expression of *Scr* in the presumptive gastric caeca, and in the posterior midgut is itself downregulated by Abd-A. In the region of the midgut which passes through parasegment 7, *dpp* upregulates *Ubx*, and *Ubx* in turn upregulates *dpp* (purified *Ubx* binds *dpp* DNA *in vitro*). The *dpp* which is upregulated by *Ubx* migrates to the gut endoderm where it is necessary for upregulation of *labial* (Panganiban *et al.*, 1990). *dpp* null mutants fail to maintain expression of the homeobox-containing gene, *zen* in the amnioserosa (Rushlow and Levine, 1990).

The conclusion from these results has been that functional *dpp* is necessary, but not sufficient, for specific aspects of the expression of homeobox-containing genes in *Drosophila*. It is not however easy to extrapolate these results to the vertebrate situation

The interaction between BMP4 and *Msx* genes does not therefore, unfortunately, fill in any missing links in a previously characterised genetic pathway.

BMPs could work in concert with FGFs to control *Msx* expression:

The fibroblast growth factors, FGFs, are another sequence-related family of diffusible cell-cell signalling molecules (Goldfarb, 1994). Nine family members have been identified; they are expressed at different, diverse sites during embryogenesis, some of which overlap with sites of *Msx* expression. *Fgf2* is expressed throughout the AER and has been shown to be able to maintain *both* Progress Zone activity and *Msx1* expression (see earlier). It is likely that BMPs and FGFs both have a role in limb development, and they may act in part by regulating *Msx* genes.

This would be interesting, because one common theme of vertebrate embryology is the interaction between TGF β type molecules with FGFs to make important developmental decisions. Often these decisions are made at sites of *Msx* expression, and it may be that the upregulation of *Msx* genes is important.

Fgf2 and TGF β are required together to induce chondrogenesis in otic vesicle mesenchyme (Frenz *et al.*, 1994) - a site of *Msx* expression.

FGF2 and TGF β induce proliferation and osteocalcin synthesis in osteoblasts (Globus *et al.*, 1988) - a site of *Msx* expression; osteocalcin has an *Msx1* consensus binding site in the promoter which is necessary for high level expression of the gene (Towler 1994).

FGF2 and TGF β influence differentiation and the commitment to melanogenesis of various neural crest derived tissues (Stocker *et al.*, 1991).

The *Xnot* gene, expressed in the *Xenopus* organiser at gastrulation, is distantly related to *Msx1*. It is induced by FGF and activin (a TGF β related molecule) in synergy (von Dassow *et al.*, 1994); hence the FGF/TGF β interaction upstream of homeobox genes may be very ancient.

Further work would elucidate whether FGFs and TGF β -related molecules commonly act via *Msx* regulation. A testable prediction of the theory is that it would be possible to induce *Msx* gene expression in the limb using beads soaked in molecules that are not normally expressed there, e.g. BMP3, BMP6, *dorsalin*.

A model for the role of *Msx* genes during vertebrate evolution:

Amphioxus expresses its *msh* gene only in the neural tube (Holland, pers. comm.); for vertebrates this is probably the ancestral site of *msh* expression. Its role there is unknown, but it may be involved in early patterning of the tube.

The early history of vertebrate evolution from a chordate ancestor was characterised by a period of genomic instability, during which many developmentally important genes were duplicated (Bird, 1995). These included the *Msx* genes, *Wnts*, *Fgfs*, *Hox* genes and probably the *Bmps* too. There were probably a very small number of amplification steps (Holland, pers. comm.), and there may have been at least one complete genome duplication. In the simplest case, if all the amplifications happened at once, a large number of identical genes would be created. (Figure 8.2A, B)

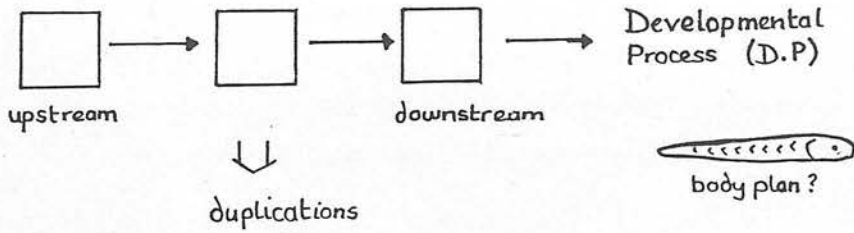
There would initially be great potential for genetic redundancy. Over time, two things are inevitable:

- (i) New genes would diverge functionally in relation to ability to do their jobs - because of random mutation in the ORF;
- (ii) New genes would diverge in expression pattern - because of changes in the promoter and gene transpositions. (e.g. *Wnt8* and *Wnt8b* in *Danio* show identical downstream gene activation and phenotypes when ectopically expressed, but show non-overlapping expression patterns during normal development (Kelly *et al.*, 1995)).

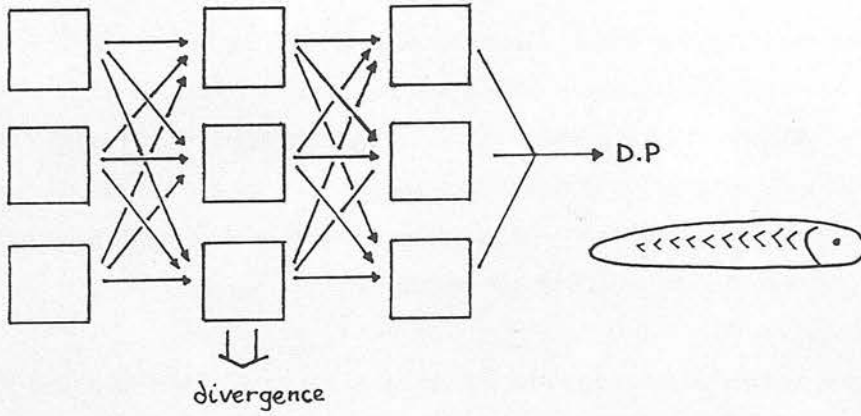
The constraints on genetic change are that the developmental programs forming the animal must get done - it must be possible to follow the arrows from the left-hand side of the matrix to 'Developmental Process'. However, when the situation seen in Figure 8.2C. is reached, the body plan can start to change.

The need to maintain a pathway through the matrix raises a problem of coevolution of the genes involved. In the situation seen in Figure 8.2D, if the shaded gene mutates, such that regulation by the two immediate upstream genes no longer occurs, then the organism is in danger of losing a part of its developmental program. There may be pressure on the genes directly upstream to coevolve with the shaded gene. If one of the genes does this such that the regulation, and hence the developmental program, is restored, the second gene may no longer have to. It is free to mutate - possibly to become a pseudogene, but maybe to do new jobs.

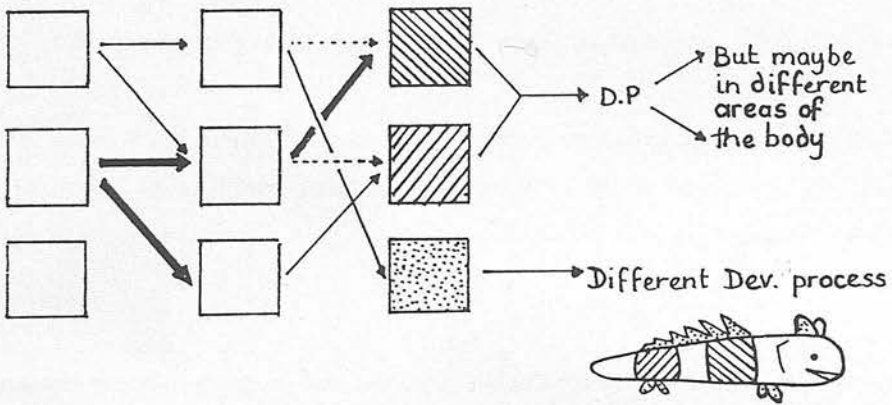
A.



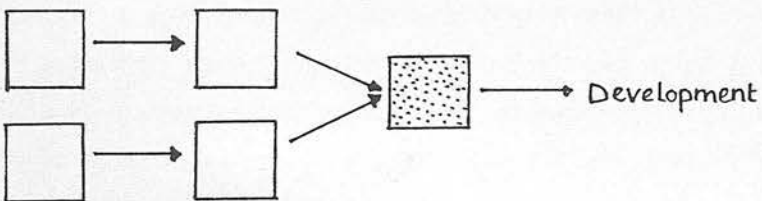
B.



C.



D.



To return to the specific *msh* case, if there was a vertebrate ancestor, controlling its *msh* gene by means of a very few FGF or TGF β -type molecules, then after the duplication steps, followed by random divergence, some of the FGF/TGF β paralogues would interact better than others with the proteins which control *Msx* genes, and better with some *Msx* genes than with others.

The genes which could still interact with *Msx* genes could themselves be undergoing promoter divergence which could lead to them being expressed in different parts of the body.

Hence, changes in the expression patterns of FGFs and TGF β s would occur with, and may be causal to, changes in the body plan. Different TGFs and FGFs would be expressed in many different locations and in distinct subsets of body tissues all the right upstream factors would be expressed at the same place, at the same time, and *Msx* genes would be turned on. This would potentially have an effect on the fate of the cells expressing them, and in an evolutionary context it would have an effect on the body plan. This is what is meant, when referring to *Msx* genes, by the phrase, 'vertebrate-specific genes doing vertebrate-specific jobs'.

Figure 8.2: (opposite)

How duplication and divergence events may have influenced vertebrate evolution (see text).

A. picturises a hypothetical ancestral genetic pathway, involving three genes (square boxes) contributing to the achievement of a developmental process (D.P.). The upstream gene (left) regulates the middle gene which regulates the downstream gene (right). This pathway may involve direct binding of the upstream proteins to the promoter of the gene immediately downstream.

After a short phase of rapid genomic change, resulting (in this case) in a triplication of the genes, the situation seen in B. occurs, with full functional interaction between all paralogues of the genes.

C.- After a period of random gene divergence, different paralogues may have different expression patterns, and differ in their ability to regulate downstream genes. (Width of the arrows represents the strength of the regulation.)

D.- see text.

Developmental decisions in vertebrate-specific structures and tissues:

Endochondrial bone in the limb has a different embryonic origin from that of the axial skeleton. Lateral plate mesenchyme-derived cells form the limb skeleton, but sclerotomal cells of the somite form that of the torso. At early stages of embryonic development, the undifferentiated lateral plate mesenchyme and the undifferentiated 'epithelial ball' somites coexist in the embryo; the lineages which these cells and their descendants will follow are separate, yet descendants of both these tissues will terminally differentiate as bone. Bone-formation is not therefore a property of a unique cell lineage (an assertion reinforced by bone-formation in the neural crest-derived mesenchyme of the head). In these cases, independent lineages of cells must independently express the correct battery of genes in the correct orders to produce the bone tissue. (The same argument could be proposed for formation of muscle tissue in disparate lineages).

There are therefore certain times during development when cells must initiate a particular gene cascade to send them down a particular developmental pathway, such as bone formation. Cells in different parts of the body, with very different lineage histories, may initiate these same cascades. The genetic basis of the ability of cells to 'wipe the slate clean' - and hence respond to differentiation signals which, because of their history, they would otherwise not do - is uncertain. I speculate that expression of *Msx* genes may, in certain, as-yet-undefined, situations, lead to the expression of downstream factors (such as members of signal transduction pathways) which enable cells to do this.

It is interesting that all known mutations in *Msx* genes cause defects in the bones of the skull. The evolution of a new tissue type, such as bone, has involved the redirection of old signals, such as *dpp*, to the transcription of a different set of downstream structural genes. The model proposed on page 221 pictures how it would be possible for duplication and sequence divergence of downstream genes to redirect the response to ancestral signals. It therefore provides a conceptual framework to explain how amplification of genes such as *msh* may have had a direct effect on the evolution of new tissue types.

It is also interesting that BMP2 can inhibit myogenesis and cause osteogenic differentiation when applied to rat C26 osteoblast precursor cell lines (Yamaguchi *et al.*, 1991). The original assay by which BMPs were discovered was that they could cause transdifferentiation of muscle to bone. There is no present reconciliation of the twin facts of the occurrence of transdifferentiation and the complete lack of 'intermediate' tissue types. Proteins such as the BMPs, and by implication the *Msx* genes, may provide the entry point for studies of how genetic pathways are perverted during the evolution of a new tissue.

3. THE pH7lacT AND pH7lacΔ3' CONSTRUCTS

The behaviour of microinjected constructs in 10T^{1/2} cells:

Most of the technical problems associated with this experiment were eventually overcome. Microinjection is a proven means of introducing DNA to a cell nucleus (Cappechi, 1980). I, as well as other workers using the same machine (Larsson *et al.*, 1995), have reliably achieved cell transfection by this method. I showed that injected 10T^{1/2} cells could express the introduced constructs, that I could graft successfully to induce the endogenous *Msx* genes, and that 10T^{1/2} cells could survive in the chicken limb.

Is expression of the pH7lacT construct induced in cells grafted underneath the AER?

A large number of experiments, described in Chapter 4, attempted to show that expression of the pH7lacT construct was induced when injected into cells which were subsequently placed under the AER, but not in those placed more proximally in the chick limb. There was some evidence that this may be the case, but it was not conclusive.

There was no convincing AER response from 10T^{1/2} cells injected with pH7lacT. Nevertheless, there was a tendency for distal grafts to show blue cells more frequently than proximal grafts. It is possible that 10T^{1/2} cells, like 3T3 cells (page 131), proliferate more quickly when placed distally than proximally, and that this is the reason why distal grafts show more blue cells.

Part of the problem with the experiments was that the reporter gene was not introduced into enough cells by microinjection, and that 10T^{1/2} cells were not successfully transfected by chemical means. Microinjection was chosen a fast means of getting DNA into cells because it had always been intended to perform the experiments with primary limb mesenchyme cells, which would require that the experiment be done as quickly as possible, before differentiation started. It was decided to persevere with 10T^{1/2} cells because of the difference in behaviour between pH7lacΔ3' and pH7lacT in these cells (which suggested that there was an interesting biological phenomenon to be investigated). While there may be problems with introducing naked DNA into eukaryotic cells (see below), I feel that the basic problem is that 10T^{1/2}s were the wrong cells to use.

It was very important to check that 10T^{1/2} cells expressed *Msx1* when grafted under the AER, but not proximally. In contrast, I found that 10T^{1/2} cells do not express *Msx* genes at all. Either they lack an element of the signalling pathway by means of which the AER signal is transduced, or the *Msx*

genes are permanently silenced in $10T^{1/2}$ cells (This would not be unprecedented, Goldhamer *et al.* (1995) found evidence, looking at the chromatin structure of a *MyoD* enhancer in $10T^{1/2}$ cells, which suggested that it was repressed by epigenetic mechanisms). It seems that $10T^{1/2}$ cells are too far removed from the *in vivo* situation to be able to give meaningful results in the grafting experiments. This is why limb mesenchyme cells would have been first choice, had the technology to transfect them been available.

On page 130 I explained the original rationale behind the choice of $10T^{1/2}$ cells. Subsequent publications provided further reasons to expect $10T^{1/2}$ cells to modulate gene expression in response to the AER signal (below).

$10T^{1/2}$ cells can differentiate in response to BMPs:

Wang *et al.* (1993) found that $10T^{1/2}$ cells can differentiate in response to BMP2. BMP2 supplied externally in the culture medium or internally, via transfection, can produce stable changes in $10T^{1/2}$ cell fate. Low concentrations of BMP2 cause differentiation into adipocytes and chondrocytes, whereas increasing the concentration leads to chondrogenic differentiation only. There are also effects on growth rate and cell density. Myoblasts are never formed, hence the differentiation pathways unleashed are not identical with those which result from application of 5-azacytidine.

The implication is that $10T^{1/2}$ cells have functional receptors and a signal transduction pathway for response to BMP2 and possibly other BMPs too. Differentiation of $10T^{1/2}$ cells can be enhanced, and their growth rate modulated, by addition of FGF4, LIF or PDGF to the culture medium, so the cells probably have receptors for these also.

BMP2 and FGF4 can probably induce *Msx1* synergistically in cultured limb mesenchyme cells (Buckland pers. comm.). In hindsight therefore, it is surprising that $10T^{1/2}$ cells cannot express *Msx* genes.

It is obvious that whilst it would be unreasonable to expect $10T^{1/2}$ cells to differentiate into limb tissues when grafted into the chicken limb, they can be induced to undergo changes in gene expression in response to external signals. Their first cousins, the 3T3 cells, can respond appropriately to *in vivo* limb-positional signals. Thus the original assumption that $10T^{1/2}$ cells were a reasonable choice for studying expression of the *lacZ* reporter constructs was well-founded.

The difference between the behaviour of pH7lacT and pH7lacΔ3' in 10T^{1/2} cells:

pH7lacΔ3' is expressed at high frequency, possibly constitutively, in 10T^{1/2} cells, whereas pH7lacT is expressed only at very low frequency. In both injected and stably transformed cells, in all situations, frequency of Δ3' expression was equal to that of pCMVβ, and greater than that of pH7lacT. pH7lacT was not abnormally toxic. The fact that some cells could strongly express active β-Gal on the promoter indicates that there was no steric interference of β-Gal activity in the chimaeric protein. Gnirke *et al.* (1993) showed that DNA constructs up to 100kb can be microinjected without shearing, so there should have been no damage to the 17kb pH7lacT during injection.

Experiments had been performed with DNA which I obtained from 4 separate plasmid preparations, and restriction enzyme analysis (such as that used to isolate the lacT insert on page 000) had shown that the integrity of the plasmid was maintained. The difference in behaviour between the two constructs cannot therefore be explained in terms of poor quality of lacT DNA.

The result suggests that there are regulatory sequences in pH7lacT, absent from pH7lacΔ3', and which have an effect in 10T^{1/2} cells. These regulatory sequences make the likelihood of a cell expressing lacT much less than the likelihood of expressing lacΔ3'. Expression of *lacZ* in any one particular cell may depend on the action of a random stochastic factor, modulated by these regulatory sequences.

The most likely sites for the extra regulatory sequences in lacT are the *MsxI* intron, the 3'UTR, or the regions 3' of the gene. There are precedents for gene regulatory sequences in each of these regions. (e.g. Gillies *et al.*, 1983; Puschel *et al.*, 1991, and other examples given earlier)

The 3'UTR and control of gene expression:

There are many examples of the 3'UTRs of message RNAs having a regulatory effect on protein production (reviewed in Decker and Parker, 1995). The 3' UTRs of *Troponin-I*, *Tropomyosin* and *α-Cardiac actin* mRNAs can activate muscle specific promoters in 10T^{1/2} cells by a currently unknown mechanism (Rastinejad and Blau, 1993). There may be autoregulatory capacity in the *MsxI* UTR which is missing in pH7lacΔ3'.

3'UTRs may, as with *fem-3* in *C. elegans*, be able to negatively regulate translation of their mRNA. They can control mRNA stability (the 3'UTR may contain sequence-specific endonucleolytic cleavage sites), or localise mRNA to a specific region of the cell - as with the *Drosophila* genes, *bicoid* and *oskar* (Macdonald and Struhl, 1988).

Behaviour of pCMV β , pH7lac Δ 3' and pH7lacT in stably transformed cell lines:

Less than 2% of cells which had genomically integrated pCMV β or pH7lac Δ 3' expressed β -Gal at detectable levels. There was still a difference ($>10\times$) between the proportion of cells which expressed these constructs and those which expressed pH7lacT. It is known that cells transfected with apparently constitutive promoters often do not constitutively express them, but I still have to ask the question, why?

Selection had been carried out in G418 sulphate. Because this selects for neomycin resistance, not for *lacZ*, it was possible that some of the cells which survived selection were those which had integrated *neo^r*, but not *lacZ*. This was intuitively unlikely, as the 1:20 ratio of *neo^r:lacZ* in the transfection mix suggested there should be very few of these cells. It was confirmed directly - subsequent PCR analysis of DNA from 20 clonal cell lines (R. Buckland, pers. comm.) showed that they were all carrying *lacZ*. Thus, although all contained the construct (or at least *lacZ*!), few of them expressed it and none of them showed a convincing induction of expression in response to signals underneath the AER.

There are several reasons why the introduced promoters may not be expressed - some of these reasons apply equally to integrated and non-integrated DNA.

- (i) It could be that the promoters which were used do not work! Expression of the constructs in injected cells had shown, however, that the promoters *do* work.
- (ii) There are some obvious conceptual problems with the expression of bacterially-produced DNA in a eukaryotic cell. The supercoiling of the introduced DNA is not defined, and may not be in a form which is accessible to the eukaryotic transcription factors. Naked DNA in the nucleus needs to bind histones and may need to attach to (undefined) structural scaffold elements in the nucleus for it to be efficiently transcribed (Stief *et al.*, 1989).
- (iii) The methylation state of the DNA after introduction is not known. Methylation of cytosine at CpG sites is a method used by vertebrates to reduce leaky transcription from genes which have been downregulated during development (possibly causing changes in the structure of the chromatin which make it unavailable for transcription factor binding) (reviewed in Cedar, 1988; Bird, 1987). Methylation status is inherited stably, due to the action of the methylase enzyme which recognises those CpG sites after replication where only one of the Cs is methylated. Methylation hence reinforces the developmental decisions which downregulate genes, although itself may not be sufficient to silence a gene (Reviewed in Alberts *et al.*, 1989). There is evidence that exogenous DNA becomes heavily methylated when introduced to the eukaryotic nucleus (Bird, 1987) so there is a possibility that this is affecting the transcription status of the introduced constructs.

(iv) There could be position effects due to integration of the introduced plasmid at a site in the genome where the local chromatin structure (see pages 233-234) or surrounding enhancer elements affect its expression. It is perhaps unlikely that this would occur with the frequency required to explain the observed behaviour of the cells.

When clonal cell lines were fixed and stained in culture, some cells were found to contain β -Gal activity and some not. This shows that expression of the constructs is not a consistent clonal property - this argues against silencing by position effect (iv), and by methylation (iii). The simplest way to ask whether methylation is having an effect on the integrated constructs is to grow the cell lines in the presence of 5-azacytidine, to ask firstly whether the frequency of expression increases, and secondly, which cell types express the constructs as the $10T^{1/2}$ s differentiate.

It seems that the decision, as to whether to express the integrated constructs or not, may balance precariously on a stoichiometric fulcrum. It also may be a cell-cycle-specific effect. Never-the-less, in common with expression of injected constructs, it is stochastically much less likely that any one cell will express lacT, than it will express lac $\Delta 3'$ or CMV β .

4. Δ H TRANSGENIC MICE

Consistent with the 4.7kb being only a part of the promoter of the endogenous gene, the Δ H transgenics show only an approximation to *Msx1*'s expression pattern. All the lines show common aspects of expression, and several of the lines show high degrees of similarity. It is likely therefore that the expression pattern represents the genuine capabilities of the transgene's promoter, rather than reflecting the action of enhancers near the integration site..

In 1990, when the first mice were made, it was thought probable that the 4.7kb would represent the whole of the *Msx1* regulatory sequence. This now seems not to be the case. Eukaryotic enhancer elements can be highly scattered (see page 245) and there are many precedents for eukaryotic promoters stretching more than 5kb upstream of the gene.

At least 2, and possibly many more, molecules can signal to cells to upregulate *Msx1*. I suggest that the the transgene may not be responding to all these molecules, and (when compared to *Msx1*) may be reacting to others at different thresholds concentrations, and to different extents.

The transgene may not be responsive to BMP4, but this is not proven:

My experiments showed neither induction nor maintenance of transgene expression by BMP4 at concentrations which induced endogenous *Msx1*. This does not necessarily mean that the transgene cannot respond to BMP4 for the reasons below.

- (a) The failure of handplate tissue to turn on (Experiment 7.02) may be taken as evidence for silencing of this tissue.
- (b) In experiment 7.03, expression was not maintained in E10.5 limb tissue, but this experiment was performed on de-epithelialised mesenchyme. The epithelium is a source of FGFs, Wnts and other BMPs and it is possible, likely even, that one of these other molecules, which would have been absent in my culture experiment, works in combination with BMP4 to maintain the transgene, and *Msx1*. Wang and Sassoon (1995) have, however, recently provided evidence that BMP4 can induce *Msx1* in cultured mouse limb mesenchyme, in the absence of other growth factors.
- (c) I was assuming that mouse mesenchyme induces *Msx1* expression in response to Bmp4, but I did not attempt to show this. Whereas it is often assumed that mouse and chicken tissue will respond in the same manner to extrinsic signals (an assumption that is supported by the grafting experiments of myself and others), there *are* differences between mouse and chicken tissue in terms of response to BMP4, which may be related to regulation of different downstream genes. Cultured mouse limb mesenchyme, in contrast to chicken tissue, does not readily chondrify in the presence of BMP4 (Reddi, 1994).

If the *lacΔ3'* transgene really could not respond to BMP4, the domains in the embryo which express *Bmp4* and the *Msx* genes would be the areas where one would expect transgene expression to be deficient. Thus, absence of transgene expression in the teeth and whisker follicles, and very weak expression in the hindbrain might support this hypothesis. However, *Bmp4* is also expressed in posterior mesoderm after gastrulation, in the epithelium and mesenchyme of the branchial arches and in the posterior of the otic vesicles - domains which also express the transgene (and *Msx1*). Like the ΔH transgenes, the *Bmp4* receptor, *ALK6*, is expressed specifically in the embryonic mandibular mesenchyme, but not in that of the maxilla (Dewulf *et al.*, 1995), which suggests that the transgene is responding to *Bmp4* (via *ALK6*) but that the response to some other extrinsic signal (or the *ALK3* pathway) is deficient. It is not therefore possible to definitively relate differences between the expression patterns of the transgene and *Msx1*, to a failure of the transgene to be activated by *Bmp4*. A more comprehensive knowledge of the activating molecules immediately upstream of *Msx1* is necessary.

It would be immediately possible to investigate the response of transgenic mesenchyme to FGFs and TGF β -related molecules (implanting beads into cultured transgenic tissue), and to ask whether BMP4 needs to work in synergy with these other molecules to induce or maintain expression of the transgene. Only when there is a clearer knowledge of the *in vivo* activators of *Msx* genes, their expression domains and the cooperative interactions between them, will it be possible to satisfactorily investigate to which, if any, extrinsic signals the transgene is unable to respond.

There is probably no response from either the *lacΔ3'* or *lacT* constructs to extrinsic signals underneath the AER:

An inductive response to extrinsic signals subjacent to the AER was not shown in the grafting experiments with $\Delta H5$ tissue, (but this will be discussed later). To my mind, the best evidence that the constructs do not show an AER response is that of all the $\Delta H5$ lines, many ΔH transient transgenic embryos, and two *lacT* transgenic mice, none show transgene expression in distal mesenchyme after E11.5 - consistent with a failure to maintain *lacZ* expression after the formation of the AER.

It is tempting to speculate that because *Bmp4* is expressed in and under the AER, that the apparent inability to respond to the AER signal may be synonymous with the apparent inability to respond to BMP4.

Candidates for the signals which maintain expression in grafted 10.5d Progress Zone tissue:

Experimentally, I showed that there are signals within the limb which could maintain expression of the transgene in E10.5 Progress Zone mesenchyme.

Grafting this tissue into a very young limb bud maintains expression during the next 24 hours, and there is good evidence that grafting underneath the anterior epithelium of an older limb does too. These are the same regions which cannot reinitiate expression in E11.5 Progress Zone tissue.

Of all the grafts which, at fixation, were in an area (in or out of the limb) where one would not expect expression of the transgene, none retained significant levels of blue. The majority of grafts which were in transgene expressing regions were blue (although it is still puzzling that some of the 'anterior' grafts apparently failed to take, and that some cells in 'positive' grafts were negative).

Maintenance of expression by mandibular epithelium was not as convincing as in the limb, and grafts of E10.5 tissue to the maxillae do not maintain expression of the transgene. Perhaps this was not surprising. Richman and Tickle (1989, 1992) and Brown *et al.*, (1993) showed that the inductive signals from the limb and facial mesenchyme were similar, but not identical, and that expression of *Msx1* is not induced when mesenchyme is grafted from the limb to distal maxillary mesenchyme. I did not attempt to show whether endogenous *Msx1* could be induced or maintained in my limb/face experiments.

The signals which affect expression of the transgene are, as I suggested previously, probably mostly those which affect endogenous *Msx1*, except that the transgene cannot respond to some of them, and that it responds to others at different threshold concentrations and to different extents.

In the very young, pre-AER, limb bud, the epithelium is the best candidate for maintaining transgene expression, since de-epithelialised E10.5 distal mesenchyme downregulates the gene within 24 hours even though the cells hold together as a clump and survive. In contrast, mesenchymal cells which retain close proximity to an epithelium in culture retain expression during the next 24 hours, even if they present an open face to the medium.

In older limbs, the anterior epithelium has been shown to release diffusible signals which induce endogenous *Msx* genes (Coelho *et al.*, 1993), and my grafts in Experiment 4.01, provided an *in vivo* demonstration of this. The anterior epithelium therefore probably has a role in the maintenance of transgene expression in the anterior of the limb bud.

It is still possible that signalling molecules passing between the anterior myogenic cells in culture also have a role, and that these signals maintain expression in grafted E10.5 Progress Zone mesenchyme. It would be possible to test this by mixing E10.5 transgenic Progress Zone mesenchyme with anterior stage 25 chicken mesenchyme, and seeing if this could rescue transgene expression after 24 hours in culture.

Whatever the nature or source of the signals, the bottom line is that E10.5 Progress Zone mesenchyme can be grafted into certain regions of the embryo which will modulate expression of the transgene. Soon after the formation of the AER, expression of the transgene is downregulated, and the same signals which modulated transgene expression at E10.5 can no longer do so.

Limb mesenchyme downregulates transgene expression to undetectable levels during development, and this is apparently irreversible:

Mesenchyme from the handplate of a W6 limb is still responsive to signals which induce expression of endogenous *Msx1* - I showed that it could, when grafted underneath the AER of a young chicken limb, subsequently express endogenous *Msx1* within the chicken's *Msx1* expressing domains (only).

In contrast, transgenic handplate mesenchyme, which is entirely negative for β -Gal activity, after placing a graft either underneath the chick AER, in very young limbs, underneath anterior epithelium, in cultured mouse limbs, or in cultured mouse facial processes, never detectably reinitiated *lacZ* expression. It seems to be entirely refractory to inductive signals.

Possible objections to these experiments are that transgene expression was initiated, but at very low levels which are not detectable after a few hours X-Gal staining, or that 18 hours in the limb may not be long enough to reinitiate transgene expression. There are two answers to these objections.

Firstly, it is clear that X-Gal is a very sensitive technique for detecting β -Gal activity. Overnight staining also failed to turn the tissue blue, which suggested there was no β -Gal present. Δ H5 tissue which is expressing *lacZ* turns intensely blue within two hours of staining.

Secondly, grafts were normally left *in situ* overnight, i.e. 16-20 hours, but some anterior grafts were left for over 24 hours. In the experiments of Davidson *et al.* (1991), induction of endogenous *Msx1* is detectable within 5 hours; presuming that the kinetics of transgene induction would be roughly the same, then I suggest that were the transgene induced, it would be expressing *lacZ* for at least 12 hours before fixation. Staining transgenic mice, which results in intense blue colouration within 2 hours, represents the accumulation of the last 24 hour's *lacZ* activity within the staining cells. (I conclude

this because β -Gal activity in the Progress Zone goes from 'intense' to zero between E10.5 (W4) and E11.5 (W6), i.e. β -Gal lingers for no longer than 24 hours in cells).

Hence, if there was induction in the grafts, it should be of the order of one half as strong as the level of expression seen in transgenic mice. Clearly, this is not the case in the grafts, and from this I conclude that grafted, non-expressing mesenchyme shows minimal, if any, response to inductive signals. This is reminiscent of the promoter being 'closed for business' in the language of the model proposed by Akam (1988) for control of homeotic genes in *Drosophila*.

A demonstration of silencing demands that the promoter can be 'open for business' and that there are signals in the embryo to which it can respond. It was, for example, possible that the transgene was turned on at gastrulation, but did not respond to any signals after that, leading to gradual loss of expressing tissues. This seemed unlikely because:

- (i) I had shown that experimental maintenance of expression was possible in E10.5 limb tissue;
- (ii) β -Gal activity disappeared at different times in different parts of the embryo - lingering in mandibles and arms until E18 whilst disappearing from the neural tube at E10;
- (ii) in some areas of the embryo, the transgene can be seen to be turned on *de novo* in non-expressing tissue e.g. somites, facial processes.

The phenomenon of the silencing or modulation of transgenes due to their genomic location is well known (reviewed in Gridley *et al.*, 1987). Because eukaryotic enhancers can work at large distances, inserted transgenes, especially those with weak or no promoters, can show expression patterns which are partly under the control of surrounding endogenous enhancers (Allen *et al.*, 1988) - this is the basis of enhancer trap experiments (Friedrich and Soriano, 1992; Joyner, 1991, review; Rossant and Hopkins, 1992) and targeted gene expression experiments (Halder *et al.*, 1995). However, one does not necessarily need to evoke ectopic genomic location as the sole explanation for possible silencing of a transgene in a specific tissue midway through embryogenesis. There are two widespread mechanisms by which a eukaryotic promoter can be rendered conformationally inaccessible to the transcriptional apparatus. These mechanisms, and how they may affect the ΔH transgene, are reviewed below.

Higher order regulators of gene expression in eukaryotic cells:

1. Heterochromatisation:

It is well known that chromosomal location *can* effect the expression of genes in both vertebrates and invertebrates, the classic examples being those revealed by position effect variegation (Hennikoff, 1990; Spradling and Karpen, 1990 - reviews). Large areas of the eukaryotic genome, such as the pericentric domains, are held in a stable compacted state (heterochromatin). Heterochromatisation is thought to occur when a large multimeric protein complex irreversibly binds DNA and folds it to such an extent that it is no longer accessible to the transcriptional apparatus.

Two features of heterochromatisation are of interest here: firstly, it is a highly heritable state - areas of the genome which are silenced in a cell are usually also silenced in the progeny of that cell, and *vice versa*; secondly, heterochromatisation appears to creep along a chromosome from proposed nucleation centres, spreading along the chromosome by cooperative binding of the elements of the protein complex (a finite pool of genetically identified *Su(var)*s, some of which are histones) until some signal on tells it to stop (Singh, 1994; Pirota and Rastelli, 1994).

Heterochromatinisation occurs in vertebrates (witness X inactivation) and can certainly effect transgenes, even those which insert in euchromatin. There may be specific DNA sequences which act as nucleation centres, but it is possible that repeat sequence DNA is a prime nucleator for heterochromatic proteins. The vast majority of normal heterochromatin consists of repeat sequence DNA, and in experimental situations (Dorer and Henikoff, 1994), it was found that heterochromatic silencing of a *white* transgene was more likely when it was inserted as a repeat array.

In mice, transgenes usually insert as a concatemer of tandem repeats (Gridley *et al.*, 1987), and Stief *et al.* (1989) found that mouse transgene expression is more likely to be modulated if the gene is present in increased copy number. In plants, silencing interactions between duplicated genes are frequently observed (Matzke *et al.*, 1987).

The mechanisms therefore exist within a mouse cell to heterochromatinise an introduced gene, whether it be a free-floating plasmid or an integrated transgene. Heterochromatisation occurs early in embryogenesis (Singh, 1994), however, and it is unlikely, however, to produce the developmentally-regulated pattern of silencing which is implicated for the $\Delta H5$ transgene. There is a second, sharper, weapon by means of which genes can be silenced. It involves a second multimeric, chromatin-binding complex formed by a different, but related, set of proteins - the *Polycomb* group.

2. Polycomb and trithorax groups - protein complexes which maintain stable promoter states.

There are two facets to correct expression of the homeotic genes - the initiation of expression and its subsequent maintenance; these two aspects can be genetically isolated. For example, homozygotes of the *Polycomb*, *Pc*, mutation of *Drosophila* initiate correct homeotic gene expression at the cellular blastoderm stage, but later die before pupation due to indiscriminate turn-on of all homeotic genes in all segments, giving a larva in which all the segments are replicas of the last abdominal segment (Paro, 1990). There are at least twenty genes which lead to a similar phenotype, of varying severities, such as *polyhomeotic* and *extra-sex combs* (Paro, 1990). Conversely, there is a group of mutations, characterised by the *trithorax* gene, in which although homeotic gene expression again initiates correctly, maintenance of gene expression does not occur (Kennison, 1993). The heritable transcriptional state of the homeotic genes appears to be controlled in part by chromatin conformation in a mechanistic parallel of heterochromatisation mechanisms (Paro, 1990).

The *Pc* protein has been cloned (Orlando and Paro, 1993). It is expressed ubiquitously throughout embryogenesis and shares a 37 amino acid 'chromodomain' with the *HPI* *Su(var)* protein. In purified extracts, *Pc* has not been shown to be able to bind DNA directly, but, in common with other members of the *Polycomb*-group, *Pc-G*, genes, antibody studies show that it associates with chromatin at many sites in the genome, specifically binding to inactive, silenced genes (Paro, 1990).

Pc-G binding is genetically separable from classic PEV. Variegation effects are not modulated by adding an extra Y chromosome, nor by *Su(var)* mutations. There are sequences in the eukaryotic genome which insulate genes from P.E.V. silencing (Chung *et al.*, 1993) e.g. *Fab-7* in the Bithorax complex. These sequences do not prevent *Pc* binding, and may allow *Pc-G* genes to act without interference from the heterochromatin proteins (Pearce, 1992).

Some promoters show variegation effects when used to drive reporter genes in transgenic flies, due to binding of *Pc-G* proteins (Fauvarque and Dura, 1993). Specific DNA sequences, PREs, necessary for the binding of *Pc-G* proteins, are known (Pirodda and Rastelli, 1994). They are probably complex, but correspond in position to known regulatory elements of both homeotic and other genes. (e.g. *bxd*, *iab-2*, *iab-3*, all contain PREs) (Singh, 1994). *Pc-G* silencing can spread considerable distances from the nucleation site, hence cooperative binding is implied, forming a multimeric protein complex which renders chromatin inaccessible to the binding of transcription factors.

The *Pc* complex can silence genes during the developmental program of the animal on a gene-by-gene basis. In a *Drosophila* cell line, *Pc* has been shown to associate with chromatin along the length of the BX-C complex, except around *Abd-B*, which is the only gene to be transcribed (Orlando and Paro, 1993).

The sequence-specificity of Pc-G binding means that all promoters which have a PRE have the potential to be silenced, even if they are transgenic, and in an ectopic chromosomal location.

Less is known about the *trithorax* group of proteins, *trx-G*, which are thought to assist binding of transcription factors by maintaining chromatin in an open structure (Mazo *et al.*, 1990; Chinwalla *et al.*, 1995). These gene products probably also form a multiprotein complex (Paro, pers. comm.), which has been shown to bind to 63 sites on polytene chromosomes, including the homeotic genes and *engrailed* (Pirrotta and Rastelli, 1994). 30 of the binding regions are shared with Pc-G proteins, and it is likely that PREs and *trithorax* response elements are closely associated, and that there is competition between the two complexes for access to promoters (Chinwalla *et al.*, 1995; Paro, pers. comm.).

Pc-G silencing and Trx-G 'opening' of promoters is a stable, transmissible state. Using Pc and Trx-G genes, it is therefore possible for a cell to turn on a subset of homeotic genes according to its position within the animal, and then to maintain the expression of these genes in all its clonal descendants once the initiating signals have gone away - the cells of an imaginal disc can be cultured for many months and will retain expression of only those homeotic genes that it was expressing when excised.

Silencing in the mammalian system:

Less is known about how Pc and *trx* type genes might work in mammals. Mice have several proteins with chromodomains, one of which, *M33*, is similar to *Polycomb* (Pearce *et al.*, 1992) and which can rescue a *Polycomb* null mutant fly (Paro, pers. comm.). There are also mammalian homologues of trithorax-type genes e.g. *HRX*, a gene commonly mutated in acute leukaemias (Gu *et al.*, 1992). It has several 'AT-hook' motifs which are characteristic of the HMG (open chromatin) proteins. The *brahma* gene shows homology to human helicases which facilitate binding of the transcription apparatus (Chinwalla *et al.*, 1995).

There is good circumstantial evidence that Pc-G and Tr-G complexes (trithoracic park?) may regulate homeotic gene expression (at least) in vertebrates.

Firstly, there is the knockout of the mouse homologue of *Psc*, *Bmi*, which showed posterior homeotic transformations (van der Lugt *et al.*, 1994; reviewed in Krumlauf, 1994) (mentioned on page 6).

Secondly, anterior boundaries of homeotic gene expression in mice are maintained for at least 4 days after their initiation (Gaunt and Singh, 1990). There is a degree of cell autonomy in maintenance of homeotic gene expression - migrating neural crest cells maintain the genetic signature of the axial

level from which they arose (e.g. Murphy and Hill, 1991) even after transplantation of the rhombomere to a different cephalic level (Guthrie *et al.*, 1991); transplanting somitic mesenchyme from the cervical to the thoracic level of the chicken alters neither the 'Hox code' of the transplanted tissue, nor the structures into which it differentiates (Gaunt and Singh, 1990). In the limb, moving tissue from the anterior margin which expresses *Hoxc6* to the non-expressing posterior margin does not result in downregulation of the gene in grafted tissue - it is as if the promoter is held open in the absence of inductive signals (Olivier *et al.*, 1990).

A picture is emerging that, during embryonic development, patterns of gene expression are set up, after which the *Pc* and *Trx* group genes move in to lock the expression state of those promoters which contain appropriate binding sequences. There is no need to assume that these 'open for business' effects will be limited to clustered genes - in *Drosophila*, *Polycomb* binds at many sites on polytene chromosomes, including *engrailed* (Pirotta and Rastelli, 1994).

The Pc-G genes may however need a window of opportunity to give them access to the chromatin. In *Drosophila*, the *extra sex combs*, *esc*, protein recognises the binding of *hb* to the *bcd* promoter and seems to facilitate nucleation of the Pc-G genes (Singh, 1994).

So it is plausible that the expression of any one homeotic gene in any one cell may be influenced not only by the presence or absence of the inducing factors appropriate to that gene, but also by whether the promoter is 'open for business' in terms of the secondary structure of the chromatin, mediated by Polycomb and Trithorax complexes and dependent on either the chromosomal location of the gene or the history of the cell's ancestors, or both.

The implications of this for the ΔH transgene:

It is a feature of the control of expression of *Msx1* that expression of the gene can be reinitiated in limb tissue which has previously downregulated it. This is in contrast to the results of my experiments with the transgene. It is almost certainly because the transgene's promoter lacks control elements which are present in that of *Msx1*.

One explanation of the failure of downregulated tissue to reinitiate expression of the transgene is that different signals may be required for reinitiation, compared to maintenance, and that the transgenic promoter lacks the elements required to respond to the former. It is not known why the signals which are sufficient to cause transcription in E10.5 limb mesenchyme are no longer sufficient for E11.5 tissue. I believe, for the following reasons, that the promoter may be silenced by a physical change such as that caused by Pc-G -type binding.

(i) *Pc*- and *Trx*-like genes probably bind the promoters of vertebrate genes. There are specific binding sequences, which might be present in the 4.7kb promoter sequence of the ΔH mice.

(ii) Because *Hoxa* and *Hoxd* expression does not develop fully until a relatively late stage in limb development, any stable locking of promoter activity is unlikely to occur until *Hoxd13* is expressed at W3/W4.

(iii) Because endogenous *Msx1* is not epigenetically repressed at this stage, there must be some reason why the transgene is. Something gives the silencing proteins their window of opportunity.

(a) Maybe this is due to abnormal methylation of the transgene.

(b) It may be that the inability to respond to AER signals leads to a lull in transcription factor activity on the promoter which gives space for the repressor complex to form. Experiment 6.14 may support this hypothesis.

(c) Because endogenous *Msx1* is not silenced after downregulation, upstream sequences, missing from the 4.7kb, may be necessary to bind trithorax-type euchromatin-maintaining genes. The absence of these could close the promoter down once active transcription stops. In this respect, the inability of the transgene to reinitiate expression in the limb would not be due to a lack of response elements for the 'extra' or different signals needed, but would be due to a lack of those elements which prevent the promoter being silenced. This would be analogous to the situation described by Chinwalla *et al.*, 1995 - a *Ubx* 22kb proximal promoter region fails to maintain gene expression, probably because it lacks a *trithorax* response element which is known to occur in the next distal 6kb.

In the case of (b) above, the repressor which binds to the promoter because of failure to respond to signals released by the AER need not be a Pc-G complex. It could be any repressor protein which needs the AER response proteins to remove it.

5. EXPRESSION OF $\Delta H5$ IN MUSCLES OF THE LIMB - *MSX* GENES COME HOME!

Expression throughout the limb mesoderm is not completely abolished at W4/5. The expression in the prospective myogenic cells of the anterior stylopod is maintained. The significance of this will now be considered.

Expression in the developing muscle is a significant feature of the pattern of the *msh* gene during fly development. It is not however a common feature of expression of the vertebrate *Msx* genes. Davidson (pers. comm.) has seen *Msx1* labelling in the developing muscles of the tongue (which are somite-derived) and there is weak labelling in the brachial muscle-forming region at the anterior of the limb (shoulder region) at E10-E12, but this is not specific to presumptive muscle cells and I have been unable to detect it subsequently in limb muscles. This is in contrast to the situation for the ΔH transgenes.

Expression of the ΔH transgene in myogenic cells of the limb bud:

$\Delta H5$ retained expression in some of the muscles of the limb until E17. All the limb muscles are derived solely from the dermamyotome of the somites at the level of the limb. As described previously, the prospective limb muscle cells of the somite are blue at E9. In a rapid phase of migration, the blue cells bud off and move into the limb, where at E10 they are lost in the blue limb mesenchyme.

This means that most, if not all, of the muscle cells are expressing *lacZ* when they leave the dermamyotome. By E11.5 (W6) however, most of these cells and their progeny are not expressing the transgene, except for a patch of cells in the anterior zeugopod and a few cells in the posterior-ventral autopod. Two questions must be addressed.

1. Are all the blue cells in the limb mesenchyme myogenic at this stage (W6)?

From the behaviour of the cells during subsequent development and in experimental situations, this seems most likely. The best way to prove this would be to cross the $\Delta H5$ line onto a *Pax3* mutant (*Splotch*) background. *Pax3* is expressed in the ventrolateral dermomyotome and in migrating myocytes until they start to differentiate; until now, *Pax3* was the only molecular marker for these cells (Bober *et al.*, 1994; Williams and Ordahl, 1994; Goulding *et al.*, 1994). In these mutants, the prospective limb muscle cells never leave the somite, and may never be specified. Hence *Splotch*/ $\Delta H5$ mice should show no blue in the limb after E11. It was by analysing expression of the

homeobox genes, *Six1*, in *Spotch* mutants that it was shown that *Six1* is expressed in limb myogenic cells (Oliver *et al.*, 1995).

The fact that these myogenic cells go on to form blue muscles (only) when transplanted into the chicken (Expt. 6.11) demonstrates that expression of β -Gal is more than just a transient effect on cells moving through the anterior domain of the limb.

2. Why do most of the myogenic cells not remain blue (and hence why do only a few muscles stain)?

Each somite contributes to several specific, non-random, muscles, and each muscle receives contribution from several somites (Beresford, 1983). The muscle to which a myogenic cell contributes is determined by its position in the limb during migration and splitting of the muscle masses. Somites are not determined to contribute to any specific muscle mass at their formation - position-specific differentiation is imposed by extrinsic signals (Ordahl and Le Douarin, 1992)

Thus, the block of blue cells which forms anteriorly and which will go on to contribute in part to 4 limb muscles, represents cells from more than one somite. The area of blue includes cells from both the dorsal and ventral muscle masses.

It is probable, therefore, that the position of the cells in the limb determines whether they will continue to express the transgene. There may be something about the microenvironment of this area of the limb which allows them to maintain expression - maybe a signal from the anterior epithelium.

The transgene in the anterior muscles may be responding to Fgf2. This is expressed in dorsal and ventral myogenic cells of the upper arm of chickens. The literature is unclear whether it is expressed in other muscles, but the implication is that most of the other limb muscles do not express the gene (Joseph-Silverstein *et al.*, 1989; Savage *et al.*, 1993).

Myogenic cells cannot be entirely dependent on signals from the anterior epithelium to maintain expression.

The position-specific expression of transgene in myogenic cells is, however, somewhat at odds with their behaviour in experimental situations, which suggested a degree of cell autonomy.

(i) Wherever they were grafted in the limb, mandible, or flank, they remained intensely blue after overnight incubation.

(ii) Although I did not see them differentiate fully into muscles in the chicken, they still behaved like muscle cells even in ectopic locations. Two days after grafting, they could be seen to be aggregating into clumps in the muscle mass of the chicken, away from the main body of the graft. In some cases they had migrated distally considerable distances away from the graft site (mirroring the behaviour of

myogenic cells reported by Gumpel-Pinot, 1984), still expressing the transgene. After 3.5 days, they were seen as elongated, fusing, myotubes within the developing muscle mass of the chicken.

These observations suggest that myogenic behaviour, and maintenance of expression of the transgene does not depend entirely on position in the limb, and there may be a degree of cell autonomy. At first sight, this hypothesis is supported by cell culture experiments, where the cells growing out in almost total isolation from other cells can continue to show strong β -Gal activity after 24 or 48 hours. However, after 5 days in culture, when differentiating myotubes, chondrocytes, adipocytes and fibroblasts can be distinguished, none of these cells are blue. If left in the mouse to 16 days, at least some of these myotubes would retain expression, so they do need some sort of a signal. A community-effect interaction between pre-differentiated myoblasts is known to be necessary for limb muscle differentiation to occur (Cossu *et al.*, 1995). Muscle cells send signals (eg. TGF β) between each other which influence the timing of their differentiation and fate (Cusella de Angelis *et al.*, 1994; Emerson Jr., 1993). Perhaps it is a rather non-specific signal, such as a mitogen, which allows expression to be maintained in these cells which, for whatever reason, have escaped the presumed wave of silencing which went through the limb.

Because limb muscle expression is a feature of many transgenic lines, I conclude that it is telling us something interesting about the promoter. Townley (1995) found a serum response element (SRE) close to the presumed transcription start site. SREs are commonly found in muscle-specific promoters (Treisman and Ammerer, 1992) and *Msx1* may therefore be expected to have the potential to be expressed in muscle lineages. The fact that this is not generally the case suggests that there are additional repressor elements elsewhere in the promoter which override any potential myogenic induction. It is only when a truncated promoter is used, possibly missing some of these repressors, that muscle expression is possible. Supporting evidence for this may be provided by a small series of five transgenic embryos, produced by Alasdair Mackenzie, which contain the *lacZ* gene in frame with the proximal 400bp of *Msx1* promoter. These mice showed varied expression patterns, amongst which the only common feature was widespread ectopic expression in skeletal muscles (pers. obs.). The implication is that the muscle-specific promoter is still there, but has been masked by repressor elements during evolution. It is only when the truncated promoter peels back the strata of 600 million years of evolutionary history that muscle expression is seen. Perhaps ontogeny really does recapitulate phylogeny.

Perspectives and future work on the expression of *Msx* genes in muscle lineages:

The differentiation of skeletal muscle involves a phase of proliferation of the myoblasts, with inhibition of differentiation under the control of the action of extrinsic and intrinsic growth factors which act to induce *Id* (*Inhibitor of differentiation*), repress *MyoD* and *Myf5* and maintain proliferation *via* expression of *fos-jun*. Only when these growth factors are removed do *MyoD* and *Myf5* become derepressed, activating *Myogenin* and causing the myoblasts to differentiate (reviewed in Emerson Jnr., 1993). Given the control of *Msx* genes by growth factors, there is potential for *Msx1* gene action in the inhibition of muscle differentiation. The experiments of Song *et al.* (1992) have already implicated expression of *Msx1* in the inhibition of myoblast differentiation, and this is supported by our observations of expression of *Msx1* (not *Msx2*) in the prospective, undifferentiated, limb myogenic cells of the somite.

Why therefore is *Msx1* never detected in the myotome of the somites or in cardiac and smooth muscle progenitors? Is there a genetic pathway, homologous between *Drosophila* muscles and vertebrate dermatome-derived muscles which is not shared with vertebrate muscles from other lineages?

This comes back to the question of what defines tissue types, and whether *Drosophila* tissues are ever really homologous to vertebrate tissues. Tissue differentiation requires the expression of certain genes in certain orders, leading to the expression of the structural genes which define the tissue. Providing that the final structural genes are expressed, there is no need for *any* conservation of the upstream pathways. Although I hope I have provided enough evidence previously to indicate that there is often a good deal of conservation, it should be realised that there is inevitably a degree of flexibility in this respect.

Without a knowledge of the stages during muscle differentiation that *Drosophila msh* is acting, or whether it has a greater role during the migration of the cells than their differentiation, it is difficult to suggest what the effect of *Msx1* expression on vertebrate limb muscle formation is. One experiment which may define this role is the expression of the *Msx1* protein on its truncated 4.7kb promoter in transgenic mice. This experiment would provide a specific strong ectopic expression in a subset of limb muscles which would directly ask the question whether *Msx1* expression inhibits myogenic differentiation in the limb.

6. *MSX1* INSUFFICIENCY IN THE ΔH MICE?

Comparing the tooth section shown in Fig. 5.3H with that of the E14.5 wild type and mutant mice in the knockout paper (Satokata and Maas, 1994) suggests that like the *Msx1* mutant mice, the 1st molar of the $\Delta H5$ mice is retarded at this stage. The $\Delta H5$ mice are not null for *Msx1*, because they do develop teeth (my thumb, pers. obs.). It is possible that the presence of the transgene within cells causes downregulation of *Msx1* expression by titrating out transcription factors and regulatory proteins which bind the promoter. The alternative explanation, that insertion of the transgene has disrupted a gene important in early tooth development, is less likely.

The quickest way to test whether the transgenic mice show downregulation of *Msx1* is to breed the transgene to homozygosity and see if there is a more severe *Msx1*-like phenotype in the homozygotes.

7. THE EFFECT OF PROMOTER TRUNCATION AND ECTOPIC CHROMOSOMAL LOCATION ON GENE EXPRESSION - WHAT THE $\Delta H5$ TRANSGENE SUGGESTS ABOUT EVOLUTION OF *MSX* GENES:

The 4.7kb *Msx1* promoter, by its expression domains (such as prolonged expression in limb muscles of transgenic mice) and behaviour in experimental situations (injection experiments, grafting experiments), has revealed interesting things about itself, and about endogenous *Msx1*. The evidence for scattering of regulatory elements, position-specific responses to extrinsic signals and the potential for epigenetic silencing suggests that the basic elements of control of the *Msx* genes are similar to that of other homeobox-containing genes, including the *Hox* family. This may suggest common regulatory mechanisms between the families. It is clear, however, that the 4.7kb does not represent the whole *Msx1* promoter.

How big is a eukaryotic promoter? Should 4.7kb be expected to hold all the regulatory elements for *Msx1* expression?

Studies in which *lacZ* has been expressed in transgenic mice under the control of the promoter regions of developmental genes has shown that regulatory sequences can reside many kilobases upstream or downstream of the gene.

Some promoters are quite small. 1.6kb of Myogenin 5' sequence is sufficient to drive *lacZ* expression in a manner which closely matches that of endogenous myogenin. (Tse-Chang *et al.*, 1992). Behringer (1993) showed that 4kb of *Hoxa4* 5' sequence was sufficient to drive an essentially normal expression pattern.

Other control sequences may be quite small, but located at large distances from the ORF. Goldhamer *et al.* (1995) showed that expression of human *MyoD* is controlled almost entirely by a 258bp sequence which resides 20kb 5' of the gene.

However, many promoters may contain enhancer sequences scattered over more than 4.7kb. Bierberich (1990) found that although 5.2kb of *Hoxc8* promoter drove relatively normal expression in the embryo up to E9.5, expression went awry after that, and that there were sequences up to 7.2kb upstream of the ORF which regulated the gene.

Puschel (1991) showed that sequences in the intron of *Hoxa7* were necessary for correct expression of the gene and Whiting *et al.* (1991) found elements 3' of *Hoxb4* which were necessary to drive expression in the neural tube.

Studies in *Drosophila* have shown that regulatory sequences for the *Antp*-class homeotic genes are scattered throughout the cluster, and this may be a reason why clustering is evolutionarily conserved (reviewed in Kennison, 1993; Ruddle *et al.*, 1994); for example, the *parabithorax* mutation of *Drosophila* causes loss of *Ubx* expression in the imaginal disc of parasegment 6 and maps to the *Ubx* intron. The *abx* mutation which leads to loss of *Ubx* expression in the imaginal disc of parasegment 5 maps to the *Ubx* 5' region. Enhancer elements responsible for subsets of the expression of the other homeotic genes have also been genetically mapped, and show that the *cis*-regulatory regions of the homeotic genes may reside as much as 100kb from the gene they are controlling (Kennison, 1993). Whiting *et al.* (above) suggested that *Hoxb4* shared regulatory elements with *Hoxb3*.

It is not known whether the homeotic genes are a special case, or whether lots of genes have widely dispersed enhancer elements. The misexpression of the c-kit ligand which leads to the *Steel-panda* and *Steel-contrasted* phenotypes is due to genomic rearrangements 115kb and 195 kb upstream of the gene, respectively. However, this is possibly due to a position effect caused by the rearrangements, rather than disruption of distant enhancer elements (Bedell *et al.*, 1995).

Conclusion:

By comparison with other genes, it is expected that *Msx1* regulatory sequences might stretch more than 4.7kb upstream, although it would be surprising if the transgene exhibited no *Msx*-like expression domains. This is, in fact, what my work has shown.

Is chromosomal location important for the regulation of *Msx* genes?

Some of the differences between the behaviour of endogenous *Msx1* in grafting experiments and that of the transgene, such as apparent early silencing in the limb, may be due to the fact that the transgene is in an ectopic chromosomal location.

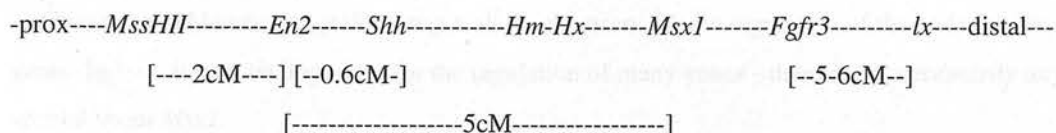
The need to co-ordinate the regulation of gene expression may explain why the HOM-C homeotic genes are clustered in vertebrates and invertebrates. Transgenes which express *lacZ* on homeotic gene promoters can show much of the expression pattern of the endogenous gene (Behringer *et al.*, 1993; Puschel *et al.*, 1991; Whiting *et al.*, 1991), but the details, timing and levels of gene expression are often abnormal (Vogels *et al.*, 1993; Schughart, 1993; Sham *et al.*, 1993).

Colinearity on the chromosome has, with some evidence (*Evx2*, page 46) been proposed as being important in imposing spatial or temporal colinearity on the expression patterns of the *Antp*-class genes. The sequential unfolding of gene expression domains - e.g. in the posterior CNS, somites, or in limbs - may be the manifestation of a change in chromatin structure creeping along the cluster, opening the promoters for business. This in turn may implicate binding of the products of the *Pc* and *trx* complex genes in the process (Duboule, 1994b).

The fact that *Drosophila* does not show temporal colinearity in its homeotic gene expression may explain why it has been able to disrupt the HOM-C complex (Duboule, 1994b).

Msx1, *Msx2* and *Msx3* show overlapping expression domains in the neural tube, as do *Msx1* and *Msx2* in early somatopleure and splanchnopleure, facial processes and the limb, which have been proposed as marking out domains along the mediolateral and dorso-ventral axes of the body (in the same way that *Hox* genes specify domains along the a/p axis) (Davidson and Hill, 1992). If this is spatial colinearity, it is in the absence of colinearity on the chromosome, since the *Msx* genes are not linked! (Monaghan *et al.*, 1992; Hill *et al.*, 1989; Bell *et al.*, 1993). It is also, according to the present level of analysis, in the absence of temporal differences in initiation of gene expression.

Chromosomal location may, never-the-less, be important. *Msx1* is found in a 'limb-related' area of mouse chromosome 5.



Msx1 - expressed in ventral ectoderm, AER, Progress Zone, interdigitally

Hm(hammertoe) - a mutation characterised by failure of interdigital cell death (Zakeri *et al.*, 1994).

Hx (hemimelic extratoes) - a limb deformity of the *luxoid* class.

(Both *Hm* and *Hx* are known to be close, but not allelic, to *Msx1*) (Robert *et al.*, 1994)

Fgfr3 - expressed in the condensations of all the limb bones. It is known to be mutant in some human craniofacial syndromes and is the best candidate for the gene at fault in *luxate*.

lx (luxate) - a mutation of the *luxoid* class.

MssHII - encodes the vertebrate homologue of a yeast protein which is part of a cell-cycling multi-unit protease complex. It is expressed in the Progress Zone of the limb, and interdigitally (C. Gordon, pers.comm.).

En2 - not expressed in the limb, though its paralogue, *En1*, is expressed in ventral ectoderm.

Shh - expressed in the ZPA (Chang *et al.*, 1994). Because *Gli3* is *extratoes*, *Xt*, (below) and its homologue, *Ci^D*, is thought to be downstream of *hh* in *Drosophila*'s genetic pathway, mutations in *Shh* and *Gli3* could in theory produce a similar phenotype in mice.

There are other, less well characterised, genes in this region, and the association of genes described above may be coincidental. However, this area of the genome has a role in limb (and craniofacial) development. The different genes may be sharing enhancer elements (albeit over unprecedentedly large distances). Certainly, if *Hm* was the site of the element responsible for interdigital expression of *Msx1*, it is a lot more than 4.7kb upstream! Chromosomal location may be important if there is a specific localised chromatin structure which brings genes within reach of their enhancers.

Hx is similar to the *Dh* (*Dominant hemimelia*) and *Xt* (*extratoes*) mutations. *Xt* is caused by a loss of function in the *Gli3* gene (a zinc finger transcription factor). *Dh* maps very close to *Gli2* (3' of *En1*) although it has yet to be shown that the *Dh* mutation affects *Gli2*. One would expect there to be a *Gli4* gene, 3' of *En2*, possibly responsible for the *Hx* mutation; this gene has been looked for, but not found (Allen, pers. comm), so it is likely that one of the surrounding genes is responsible for *Hx*. A non-*Gli* gene which produces the same phenotype as a *Gli* gene would almost certainly be involved in the same genetic pathway - the implication is that genes may be clustered because they are in the

same genetic pathway, and this would only happen were it important to the regulation or function of the pathway.

Chromosomal location therefore may well be important for the regulation of the endogenous *Msx1* gene. Indeed, it may be important for the regulation of many genes - there is not necessarily anything special about *Msx1*.

The eukaryotic genome is more than just a string of beads. The genes are part of structured chromosomes, which are part of a structured nucleus. An injected transgenic construct, like a lost stranger in a new town, is not very discriminating in choosing its new friends. Bewildered amongst a sea of chromatin, the naive promoter inserts into the genome, and having slept with the dogs it wakes with the fleas. Little wonder that only the strongest, most self-assured, most *whole*, promoter could ever hope to remain uncorrupted by its bedfellows in its Strange New World.

Gene transposition was almost certainly a prime mover in evolution. Genes involved in developmental processes moved around and changed expression patterns by promoter rearrangement and by coming under the influence of new regulatory elements at their new (insertion) site. There is no conceptual difference between the ectopic insertion of a man-made construct and that of a transposing endogenous gene. If the potential mechanisms of vertebrate evolution proposed in this thesis are to be believed it is necessary to be able to relate gross genomic changes, involving gene transposition and promoter rearrangement, to observed differences in the expression of paralogous genes.

The expression of the ΔH transgene allows us to start to examine how the expression pattern of a gene may change after one of these transposon-rearrangement events. The overriding impression from looking at the ΔH transgenes is that they still show an expression pattern which is *Msx*-like; expression is almost entirely restricted to a set of domains where one or both of the other *Msx* genes is expressed. (If an *Msx4* gene showed this pattern, no eyebrows would be raised). The conclusion is that a single gene rearrangement event could produce the different-but-overlapping relationship between the expression of *Msx1* and *Msx2*, and other genes (such as the *Hox* paralogues).

What the ΔH transgenes do not commonly show, however, is expression in completely new areas of the body - in the manner of the model proposed in Figure 8.2. This may represent a problem with doing the experiment so late in the evolutionary history of the vertebrates, and in the absence of rearrangements of the upstream controlling genes. Perhaps gene rearrangements on a larger scale, in a simpler organism whose developmental program was not so 'locked' as a modern vertebrate, may be necessary to cause evolution of neomorphic structures.

If, early in the vertebrate line, an *msh* gene with only 4.7kb of promoter had transposed to a new genomic location, it would have changed the expression domains of the *Msx* genes and could have created a monster.

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The next step in investigating the role of the changing pattern of *Msx* genes in vertebrate evolution would be to express the *Msx1* cDNA on the truncated 4.7kb promoter. This would provide a potential opportunity to relate a defined change in *Msx1* expression to a phenotype. Hopefully, by creating our monster, we could see what the *Msx* genes are doing in ontogeny, and infer what they have done in evolutionary history. By expressing *Msx1* on a truncated 4.7kb promoter in transgenic mice, we, His subjects, really would be looking into the mind of God!

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Just like the *Pied Piper* led rats through the streets, we dance like marionettes swaying to the symphony of destruction.

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